

From the DEPARTMENT OF MEDICINE, HUDDINGE

Karolinska Institutet, Stockholm, Sweden

ON OSMOLALITY AND SPERM FUNCTION DURING PROCESSING FOR ASSISTED REPRODUCTION

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ANOVA, Unit of Endocrinology On Osmolality and Sperm Function During Processing for Assisted Reproduction

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Stockholm 2020

I want to dedicate this thesis to my extraordinary husband and three children that have stood by me through all the hard work that has gone into this.

ABSTRACT

Deep basic knowledge about sperm physiology is relevant and important to optimize the outcome of procedures used during Assisted Reproductive Technologies (ART) to select spermatozoa for fertilization. More specifically, this study examined osmolality changes and consequences for sperm motility and sperm selection in the laboratory. What kind of environmental changes occur and what challenges must the spermatozoa endure after leaving the body? How do these challenges affect the spermatozoa's functions, fertilizing potential and the make-up of the genetic material they will deliver to the oocyte?

In study I, the objective was to measure the changes in osmolality that occur after collecting the ejaculate in the laboratory. After ejaculation, the sample is mixed in order to make it homogenous. This will cause the different fractions that make up the semen sample to mix. A total of 348 individual ejaculates, 5 split ejaculates and 6 ejaculate pools were studied, and it appeared that there was an individual pattern of change in osmolality over time. At 3 hours after the ejaculation, the change in osmolality ranged from 2 mOsm/kg to 164 mOsm/kg. Furthermore, it was evident that the change in osmolality was temperature dependent. Samples stored at 37°C increased significantly more in osmolality than samples stored at 18-22°C, than samples stored at 4-7°C and than samples stored at -20°C. Denaturising temperature (100°C) blocked any further increment in osmolality. One probable cause of the increase in osmolality is that the enzymes, which are abundant in the prostatic fluid, are degrading macro-molecules, such as the proteins that are abundant in the seminal vesicular fluid. When these two secretions are mixed, the enzymatic degradation can start (Mann and Lutwak-Mann, 1981).

In study II, the markers for the different fractions of the ejaculate were measured in order to relate to the change in osmolality. As well as containing high levels of proteins, the seminal vesicular fluid also contains relatively high levels of fructose. Similarly, the prostatic fluid contains high levels of zinc. It was shown that 19% of the variation in semen osmolality covaried with the relative contribution of the prostatic fluid marker, zinc, and the seminal vesicular marker, fructose, while the epididymal marker neutral α -glucosidase did not covary. Furthermore, the results show that after removing sperm from the ejaculate, the osmolality still increased, thus, the sperm did not have an effect on the increase.

In addition to the challenge of the osmotic increase occurring in the ejaculate, the preparation of the sperm for ART presents yet another challenge. Most commercial sperm preparation media, such as density gradients or swim-up media have an adjusted osmolality of 290-300mosm/kg. Thus, depending on the individual increase in osmolality of the samples, the sperm will be exposed to varying sudden decreases in osmolality during preparation.

In study III and IV, it was examined how a hypo-osmotic challenge could affect sperm motility and the outcome of sperm selection when using density gradient centrifugation. Sperm motility was assessed by Computer Assisted Sperm Analysis (CASA). When the spermatozoon was exposed to a sudden decrease in osmolality, it took up water and swelled,

causing the tail to coil and fold. This in turn, resulted in a decreased motility (VCL) with as much as 20%. Furthermore, it appears that the greater the decrease in osmolality, the lower the yield was after selection of spermatozoa by density gradient centrifugation.

In contrast, with further investigation, it was shown that the DNA-Fragmentation-Index (DFI), measured by flow cytometry of acridine-orange stained spermatozoa was not affected by longer incubation times. However, spermatozoa ejaculated directly into a buffer had lower values for DFI% compared to samples diluted with buffer shortly after ejaculation.

The negative effect on the yield was eliminated when the ejaculate was diluted soon after ejaculation or collected directly in a buffered solution.

Since the increase in osmolality in vitro is so variable, one standardized procedure for sperm preparation would not work for all ejaculates. However, if increasing osmolality can be minimized by early dilution of all samples, then the negative effects can in large be eliminated.

SVENSK SAMMANFATTNING

Specifik och grundläggande kunskap om spermiers fysiologi är relevant och viktig för resultaten av de olika behandlingarna inom området *In vitro*-fertilisering eller provrörsbefruktnings. Att man kan vara säker på att man under processen inte skadar de manliga könscellerna och att man selekterar de spermier som kommer att ge en god chans för graviditet och friska barn. Mer specifikt undersöker denna studie vad som händer i ejakulatet efter det har samlats in på laboratoriet. Vilken typ av förändringar uppstår i spermernas miljö, i ejakulatet, och vilka utmaningar ställs spermerna inför efter att ha lämnat kroppen och under de behandlingsprocesser som de går igenom inför fertilisering? Hur påverkar dessa utmaningar spermernas viktigaste funktioner, rörelsemönster och sammansättningen av de genetiska material som dem är ämnade att leverera till äggcellen?

I studie I var målet att mäta de förändringar i osmolalitet som sker efter att ha samlat in ejakulatet i laboratoriet. Ejakulatet blandas noga för att göra det homogent och därmed kunna analysera det på ett bra sätt och i och med detta så blandas de olika fraktionerna som utgör spermaprovet. Totalt mättes osmolalitet på 348 enskilda ejakulat, 5 split ejakulat och 7 ejakulatpooler och det visade sig att det finns ett individuellt mönster av förändring i osmolalitet under tiden provet står. Vid 3 timmar efter ejakulation varierade förändringen i osmolalitet från 2 mOsm/kg till 164 mOsm/kg. Dessutom var det uppenbart att förändringen i osmolalitet varierade beroende på vilken temperatur prover förvarades i. Prover som förvarades vid 37°C ökade betydligt mer i osmolalitet än prover som förvarats vid 18-22°C och de ökade mer än de som förvarats vid 4-7°C och minst ökade de som förvarats vid -20 till -22°C. När ett prov delas upp i två delar och en del utsätts för 100°C så avstannar ökningen i osmolalitet helt, medan den andra delen av provet som förvarats vid 37°C fortsätter att öka. En trolig orsak till denna ökning av antalet partiklar är att de enzymer som finns i rikliga mängder i prostatasekretet bryter ner de makromolekyler, såsom proteiner som är kända för att finnas i rikliga mängder i sädeblåsesekretet. När dessa två sekret blandas så kan den enzymatiska nedbrytningen börja (Mann & Lutwak-Mann, 1981).

I studie II mättes de biologiska markörerna för de olika fraktionerna av ejakulatet för att kunna jämföra dem med förändringen i osmolalitet. Förutom att sädeblåsesekretet innehåller höga nivåer av proteiner så innehåller den också relativt höga koncentrationer av fruktos. På samma sätt innehåller prostatasekretet höga koncentrationer av zink. Det visade sig att 19% av variationen i osmolalitet samvarierade med det relativa bidraget av zink, medan markören för sädeblåsesekretet, fruktos, inte samvarierade. Dessutom visar resultaten att efter att spermier har tagits bort från ejakulatet så ökar fortfarande osmolaliteten, alltså hade spermerna inte någon effekt på ökningen.

Förutom utmaningen med den initiala osmotiska ökningen så utsätts spermerna för ännu en utmaning då de prepareras inför ART. De flesta kommersiella prepareringsmedier för ejakulat, såsom densitetsgradienter eller swim-up medier, har en isotonisk osmolalitet på 290-300mosm/kg. Beroende på den initiala ökningen i osmolalitet i proverna kommer

spermierna att utsättas för varierande plötsliga minskningar av osmolalitet när de möter preparerings medierna.

I studie III och IV undersöktes det hur den hypoosmotiska utmaningen kan påverka spermie rörlighet och resultatet av selekteringen med densitets gradientcentrifugering. Spermierörlighet bedömdes med hjälp av Computer Assisted Sperm Analysis (CASA).

När spermatozoon utsätts för en plötslig minskning av osmolalitet tar den upp vatten och sväller vilket orsakar att svansen att rullar sig. Detta i sin tur resulterade i en minskning av rörligheten (VCL) med så mycket som 20%. Dessutom pekar resultaten åt att desto större hypoosmotiskt förändring spermierna utsätts för desto lägre yield (% spermier) får man vid en densitets gradientcentrifugering inför ART. Däremot, vid fortsatt utredning visade det sig att DNA Fragmenterings Index (DFI), mätt med flöde cytometri av acridine orange färgade spermier inte påverkades av de längre förvaringstiderna och den ökande osmolaliteten.

Den negativa effekten på % yield eliminerades när ejakulatet späddes strax efter ejakulation eller samlades in direkt i en buffrad saltlösning. Eftersom ökningen i osmolalitet *in vitro* är så varierande kommer inte ett standardiserat protokoll för preparering och selektering av spermier att fungera för alla prover. Om man genom tidig utspädning av alla prover minimerar ökningen i osmolalitet kan de negativa effekterna nästan elimineras.

PREFACE

Dr. Peter Sjöblom was one of my co-supervisors. He sadly passed away on the 9th of October 2012, in Nottingham England where he was working as the Scientific Director and Unit Manager at the IVF Unit NURTURE. We had planned to do the last part of this thesis together with Peter and I was really looking forward to that. I met Peter when I was just a child, he was a friend of my father, Paul Holmes. They met during their time in Uppsala and they later worked together at the IVF unit at Sahlgrenska Hospital. Peter then had become a true friend of our family. Peter and my father were part of the team that did the first IVF treatment in Sweden and continued on to establish IVF in Sweden. I always knew Peter as a very kind, considerate and unselfish person who was very well liked by everyone. He was also a very successful and knowledgeable clinical embryologist. He has been sadly missed as a co-supervisor and will continue to be missed. However, we are very grateful that he was part of our team for a few years at least.

ANOVA, Karolinska University Hospital, March 9th, 2020

Emma Holmes

LIST OF SCIENTIFIC PAPERS

This thesis is based on the following original articles and manuscripts. They will be referred to in the text by their Roman numeral (I-IV).

- I. **Holmes E., Björndahl L., Kvist U.**
Post-ejaculatory increase in human semen osmolality *in vitro*.
Andrologia. 2019 Aug; Vol. 51 (7): e13311
- II. **Holmes E., Björndahl L., Kvist U.**
Possible factors influencing post-ejaculatory changes of the osmolality of human semen *in vitro*
Andrologia. 2019 Dec;51(11): e13443.
- III. **Holmes E., Björndahl L., Kvist U.**
Hypotonic challenge reduces human sperm motility through coiling and folding of the tail
Submitted, first revision is being reviewed
- IV. **Holmes E., Houska Petr., Björndahl L., Kvist U.**
Semen storage affects sperm selection by gradient centrifugation
Manuscript

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LIST OF ABBREVIATIONS

ART	Assisted Reproductive Technologies
ATP	Adenosine Triphosphate
AQP3	Aquaporin-3
CASA	Computer Assisted Sperm Analysis
DFI	DNA Fragmentation Index
ESHRE	European Society of Human Reproduction and Embryology
HDS	High DNA Stainable sperm
HSA	Human Serum Albumin
ICSI	Intra Cytoplasmic Sperm Injection
IVF	<i>In vitro</i> fertilisation
OXPhos	Oxidative phosphorylation
PSA	Prostate-specific antigen
RVD	Regulatory Volume Decrease
SCSA TM	Sperm Chromatin Structure Assay
VCL	Curvilinear Velocity
VAP	Average Path Velocity
VSL	Straight Line Velocity
WHO	World Health Organization

THESIS AT A GLANCE

	AIM	METHOD/DESIGN	RESULTS	CONCLUSION
I	To investigate the changes in osmolality of human semen that may occur <i>in vitro</i> after ejaculation and during procedures necessary for ART.	Experimental study with 86 different osmolality measurements on semen samples stored <i>in vitro</i> at different times and temperatures. Osmolality was measured by freeze-point depression.	There was an increase in osmolality after liquefaction, and the degree of increase varied greatly between samples. Osmolality rose with increasing temperature, and the progressive increase was blocked by denaturising temperature.	Spermatozoa in each individual semen sample experienced a highly variable environment <i>in vitro</i> with respect to osmolality. This may be of importance regarding the handling of semen samples for the outcome of ART.
II	To investigate possible factors that influence the increase in semen osmolality <i>in vitro</i> .	Experimental study with 195 individual semen samples with or without sperm measured for osmolality after storage <i>in vitro</i> . Addition of enzyme inhibitor to semen to see if increment in osmolality stopped. Semen samples were diluted to see if increment in osmolality was affected.	The increase in osmolality during storage is not related to the presence of sperm, or anything leaking from them. The increase is however related to zinc concentration but not fructose or α -glucosidase. Enzyme inhibitors stopped the increment in osmolality and dilution significantly reduced the increment.	Factors that influence the increase in osmolality includes, those originating from the prostatic secretion (Zn), they are of enzymatic character and their affect can be reduced by dilution of the sample.
III	To investigate how human spermatozoa that have adapted to an increased osmolality react when exposed to media that have lower osmolality than the liquefied semen.	Experimental study with 22 individual semen samples exposed to hypo and hyperosmotic challenges in order to see how this effects motility and sperm tail morphology. Motility was measured by CASA.	A decreased osmolality (hypo) influence the motility pattern of the sperm. Sperm that have adapted to a high osmolality and then are exposed to a low will have a decreased motility and tail morphology will be negatively affected.	During normal handling routines in the lab, sperm may be exposed to osmotic challenges that decrease motility and cause irreversible changes to the tail morphology.
IV	To investigate whether the yield of sperm after density gradient centrifugation is affected by increased osmolality during storage followed by a hypoosmotic challenge. An additional aim was to examine if DNA integrity of the spermatozoa is affected by an increased osmolality in the ejaculate	Experimental study with 30 individual semen samples, % yield was calculated and DFI% was measured after exposure to increasing osmolalities during storage followed by hypoosmotic challenge during density gradient selection. This was compared to diluted samples and samples collected in buffered salt solution.	A decrease in % yield was observed with increased storage time and increasing osmolality. These effects were counteracted by early dilution of the samples or collection directly into a buffered salt solution.	Early dilution of samples or direct collection into a buffered salt solution can counteract negative effects that are caused by osmotic challenges that occur during handling and processing of ejaculates in the laboratory.

1 INTRODUCTION

To maintain normal cell functions, it is necessary for cells to retain the volume and ion composition inside the cell membrane. Spermatozoa have a higher water permeability relative to other mammalian cell types (Curry et al 1995) (Noiles et al 1993) (Noiles et al 1997). It has been concluded that the membrane channels responsible for the high water permeability must be pores (Drevius 1971). In recent years the understanding of water permeability in spermatozoa has made great progress since the discovery of aquaporins, water specific membrane channel proteins (King et al, 2004). Aquaporin-3 (AQP3) has been located in sperm of several species including human (Chen et al 2011).

Sperm cells are unique in that they leave the homeostatically controlled environment in the body and rely on their own mechanisms for volume regulation. In response to a change in cell volume a mammalian sperm will activate processes to restore their cellular volume, so called Regulatory Volume Decrease (RVD). This process will protect the sperm from irreversible damage due to swelling. It appears that mammalian sperm acquire this function during their transport through the epididymis (Cooper and Yeung, 2003; Petrunkina et al 2005; Yeung, et al. 2006). This function is especially evident in the cryopreservation of sperm (Petrunkina 2005)

1.1 HANDLING OF THE EJACULATE DURING ART

During *in vivo* conditions, the most fertile and progressively motile sperm are separated from the seminal fluid in the female genital tract. This is achieved by an active migration through the cervix and the cervical mucus (Mortimer 1989). During the migration the sperm go through capacitation, one sign of which can be the acrosome reaction; these events are necessary for fertilization to take place (Yanagimachi 1988). In the ART laboratory this separation and selection must be imitated meanwhile considering the artificial nature of the process. The ejaculate, as it is when collected in the laboratory does not exist under *in vivo* conditions and this is important to consider (Björndahl and Kvist 2003).

The method chosen for preparation and for selection of sperm is based on the diagnosed cause for infertility of the couple and the characteristics of the individual ejaculate. According to the Guidelines for good practise in the IVF laboratories (ESHRE 2015) there should always be a semen analysis performed according to the World Health Organization Laboratory Manual for the Examination of Human Semen (WHO 2010) prior to treatment. The goal of the election method is to concentrate and select the active and motile spermatozoa and at the same time remove all the seminal plasma and abnormal sperm. Furthermore, the preparation and the selection method should be easy to use and practical, cost-effective and overall sustainable, and it should not cause any damage to the sperm or alter them in anyway (Henkel 2003). Today there are several different methods for achieving this. The most commonly used methods are different types of migration techniques, density gradient centrifugation, and filtration techniques. The migration techniques such as the swim-up method (Mahadevan and Baker 1984) are reliant on the self propelling properties of the sperm while the density

gradient centrifugation separates based on the buoyant density of the sperm (Bolton 1986, Pousette et al. 1986). The filtration techniques are based on different filtration matrixes through which the sperm are filtered (Henkel 2003). More recently separation based on electric charge and different molecular binding has been introduced to the field (Henkel 2012). In this thesis the only method used for sperm separation is the density gradient centrifugation.

It is further stated in the ESHRE 2015 guidelines that the procedure for sperm preparation must be performed under conditions that maximize the control of temperature and pH. This in order to avoid pH and temperature fluctuations for the spermatozoa since the media often used is bicarbonate buffered and the temperatures in the laminar flow can fluctuate. After preparation the sperm suspension should be kept at 37°C and at the right pH for optimal capacitation to occur. Thus, the importance of controlling for temperature and pH is an essential part of the work in the ART laboratory. Sperm preparations must be protected from extreme temperatures (Mortimer, 2005). Generally excessive centrifugation should be avoided (WHO 2010)

The guidelines mention many properties that need to be controlled during handling of semen from ART however, there is not yet any mention of controlling or avoiding changes in osmolality that occur in the sample

1.2 THE BIOLOGY OF THE HUMAN EJACULATE

The spermatozoon

The spermatozoon is a messenger cell that has evolved to deliver unique paternal messages to the oocyte thereby making possible the creation of healthy children and grandchildren. The main messages it delivers are 1.) activation of the oocyte 2.) an intact paternal DNA 3.) the paternal centriole for future mitosis of the offspring and 4.) factors to secure its nutrition by initiation of a placenta.

The spermatozoa have evolved specifically to perform these tasks. The sperm is composed of a head attached to a tail all enclosed by a single plasma membrane. In addition, the proximal part of the tail is composed of different regions. The neck region, where the head attaches to the tail, contains two centrioles, from one of which the axoneme of the tail is created while the other will be transferred to the zygote for future cell divisions (Avidor-Reiss et al. 2019). Distal to the neck is the midpiece with the mitochondrial sheath which contains a chain of 8-20 specialized and structurally reinforced mitochondria. The mitochondria have circular mitochondrial DNA. Distal to the midpiece is the tail/principal piece followed by the tail tip. The region that connects the midpiece and the tail is called the annulus which has recently been described in the mouse (Guan et al. 2009) and the human (Barratt et al 2009).

In the center of the tail is the axoneme which consists of a central pair of tubuli connected to 9 peripheral pairs. One of the tubuli A in each pair has dynein arms, that can climb or slide on the other tubule B in the pair; this is what causes the tail to bend and is the basis for sperm

motility (Afzelius 1959). In the principal piece the axoneme is surrounded by reinforcing structures that is the nine outer dense fibers which in turn are interconnected by the ribs of the fibre sheath. The outer dense fibres contain enzymes for glycolysis which provides the main source of ATP for sperm tail movement and motility. Read more about the ATP in sperm metabolism.

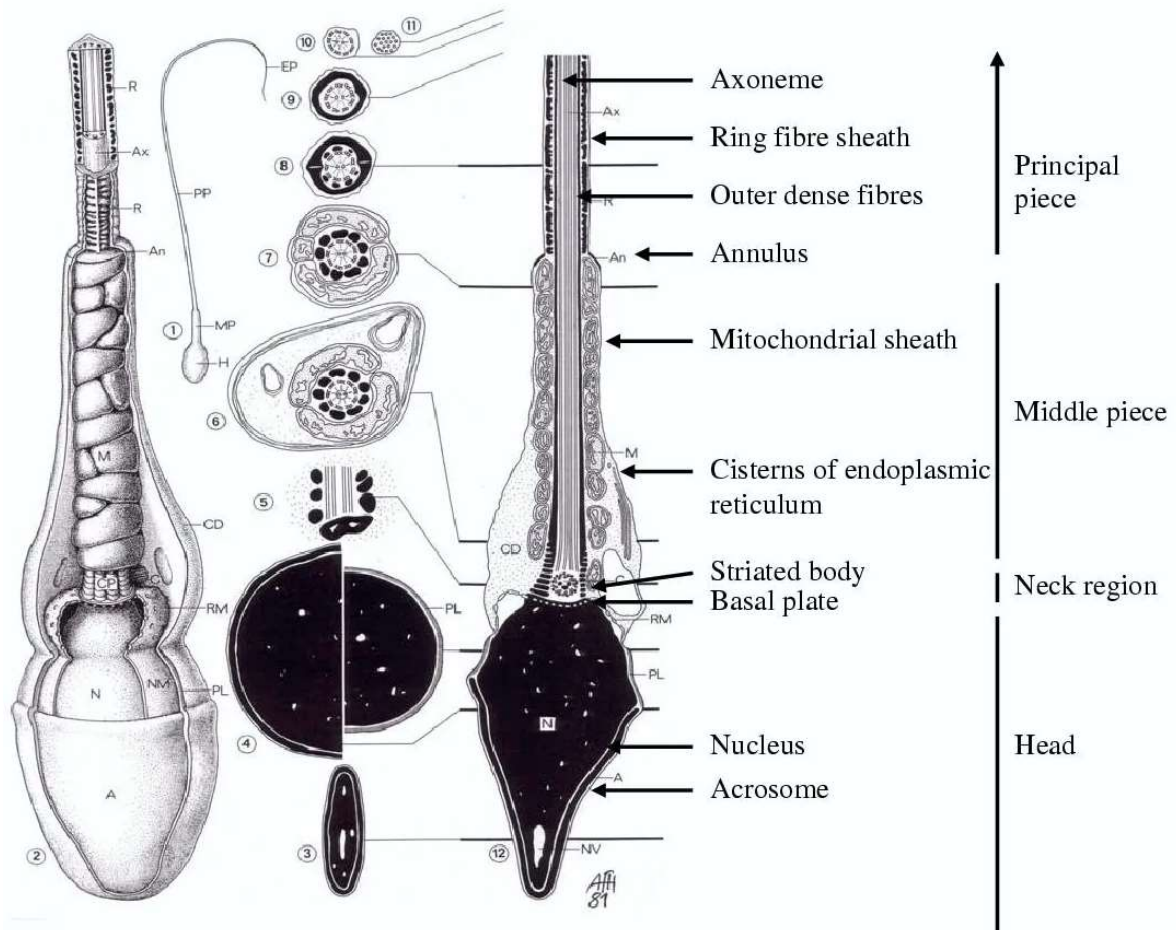


Figure 1. The anatomy of the spermatozoon (© Holstein et al; licensee BioMed Central Ltd. 2003. <http://www.rbej.com/content/1/1/107>)

The sperm plasma membrane

Maintaining fluid homeostasis is crucial for both development and function of spermatozoa. Homeostasis is maintained by means of different channels in the sperm plasma membrane. The structure of the plasma membrane follows the typical mosaic fluid model the same as any other cell (Singer and Nicholson 1972). The membrane is made up of lipids and proteins, where the lipids are dominated by phospholipids but also contain neutral lipids and glycolipids. The proportion of the lipids seems to be even species specific. The proteins of the plasma membrane are either adsorbed to extracellular sites or integrated as transmembrane proteins (Singer and Nicholson 1972, Haden et al 2000). The membrane receptors and channels responsible for maintaining homeostasis and interactions are made up of integrated or structural membrane

proteins. The adsorbed membrane proteins act as decapacitation factors and immune regulators (Rodriguez-Martinez 1998, Rodriguez-Martinez 2011). These membrane proteins are often removed during washing of the sperm *in vitro* and by the uterine secretions *in vivo* (Yudin et al. 2005). A significant proportion of the ATP expenditure of the sperm is used to keep the membrane intact and particularly the function of the channels and receptors in the membrane. Some of the most important parts of the regulation by the membrane channels and receptors are volume regulation, via aquaporins (AQP) (Yeung et al 2010; Chen and Duan 2011), intracellular calcium (Ca^{2+}) levels (via calcium channels known as CatSper (Lishko et al 2011), pH regulation by enzymes in the membrane (Litvin et al 2003), sperm-oocyte interaction and motility via CatSper and opioid receptors (Kirkman-brown et al. 2004, Tamburrino 2014, Agirregoitia 2006; 2012). What also makes this membrane specific to the sperm cell is that it can be subdivided into several domains of different morphological and functional properties See picture below (figure 2).

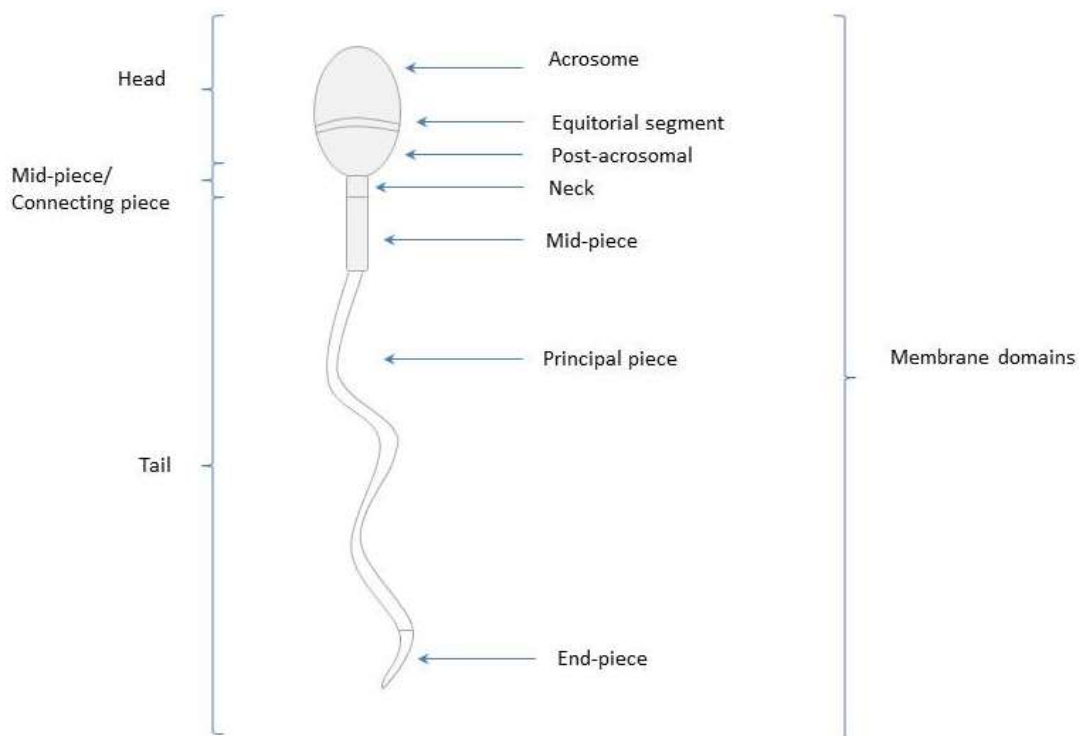


Figure 2. Diagram of the membrane domains of human spermatozoa.

The membrane consists of one layer that covers the sperm from head to tail. Furthermore, there is one nuclear envelope surrounding the nucleus and between the cell membrane and the nuclear envelope yet another membrane enclosing the acrosome which is a specialized vesicle originating from the Golgi apparatus.

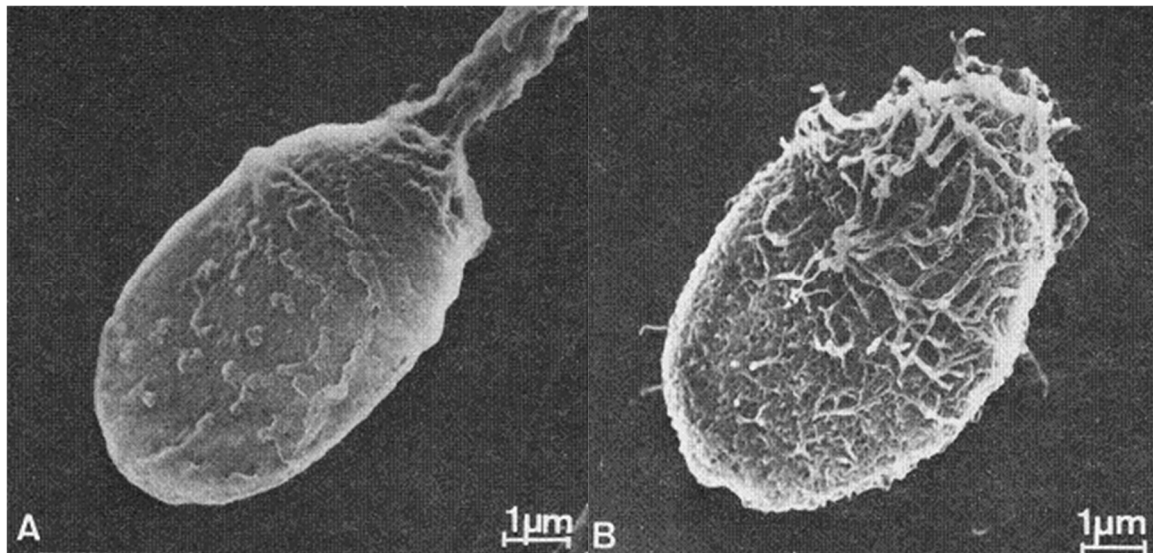


Figure 3. SEM of Sperm head with (A) and without (B) membranes. Ulrik Kvist and Lennart Nilsson 1980.

Sperm metabolism

In order to maintain homeostasis, there are two basic processes that must function in the sperm, interpreting signals received via the plasma membrane and maintaining volume and motility. These processes are active and require large amounts of adenosine triphosphate (ATP). There has been a lot of discussion whether glycolysis or oxidative phosphorylation (OXPhos) is of most importance but the latest research indicates that there are multiple sources of ATP just as in many other cell types (Ford 2006, Ruiz-Pesini et al 2007). Whenever O_2 is available and there is a restricted amount of glucose and carbohydrate residues for glycolysis, cells rely on the very efficient oxidative phosphorylation in the mitochondria.

Fetal events preceding Spermatogenesis

Already in the embryo and early fetus preparations are being made for spermatogenesis. Immature germ cells originating in the epiblasts reside first in the yolk sac and then migrate from the yolk sac and colonize the seminiferous cords in the primordial gonads (testes) where they become gonocytes that eventually develop to the spermatogonia. All this happens during the first 18 weeks in the fetus. The somatic Sertoli cells together with the spermatogonia work as a unit later during sperm production. The seminiferous cords will be channelized and become tubules at puberty. Other cells inside the seminiferous cords, specifically the Sertoli cells, will also start final proliferation at puberty.

Spermatogenesis

The process of germ cell development in the male is called spermatogenesis. In the human male the onset of spermatogenesis occurs at puberty and then continues until old age. The initiation is controlled by a hormonal interaction between the hypothalamus (GnRH), pituitary glands (FSH, LH), the Sertoli cells (Inhibin B) and the Leydig cells (Testosterone). The initial stages

of spermatogenesis start at the walls of the seminiferous tubules and proceed inwards in a centripetal direction to the innermost part, the lumen to produce immature sperm.

There are two types of spermatogonia type A, A dark (Ad) and A pale (Ap). Both of which are stem cells and are continuously renewed by mitotic division. While Ad is mainly at rest Ap will undergo two mitotic divisions into four type B spermatogonia. This process is called spermatocytogenesis and is identified by the exponential increase in number of spermatozoa. Each of the type B spermatogonia differentiates into four primary spermatocytes this means that one type A spermatogonia will result in 4 primary spermatocytes.

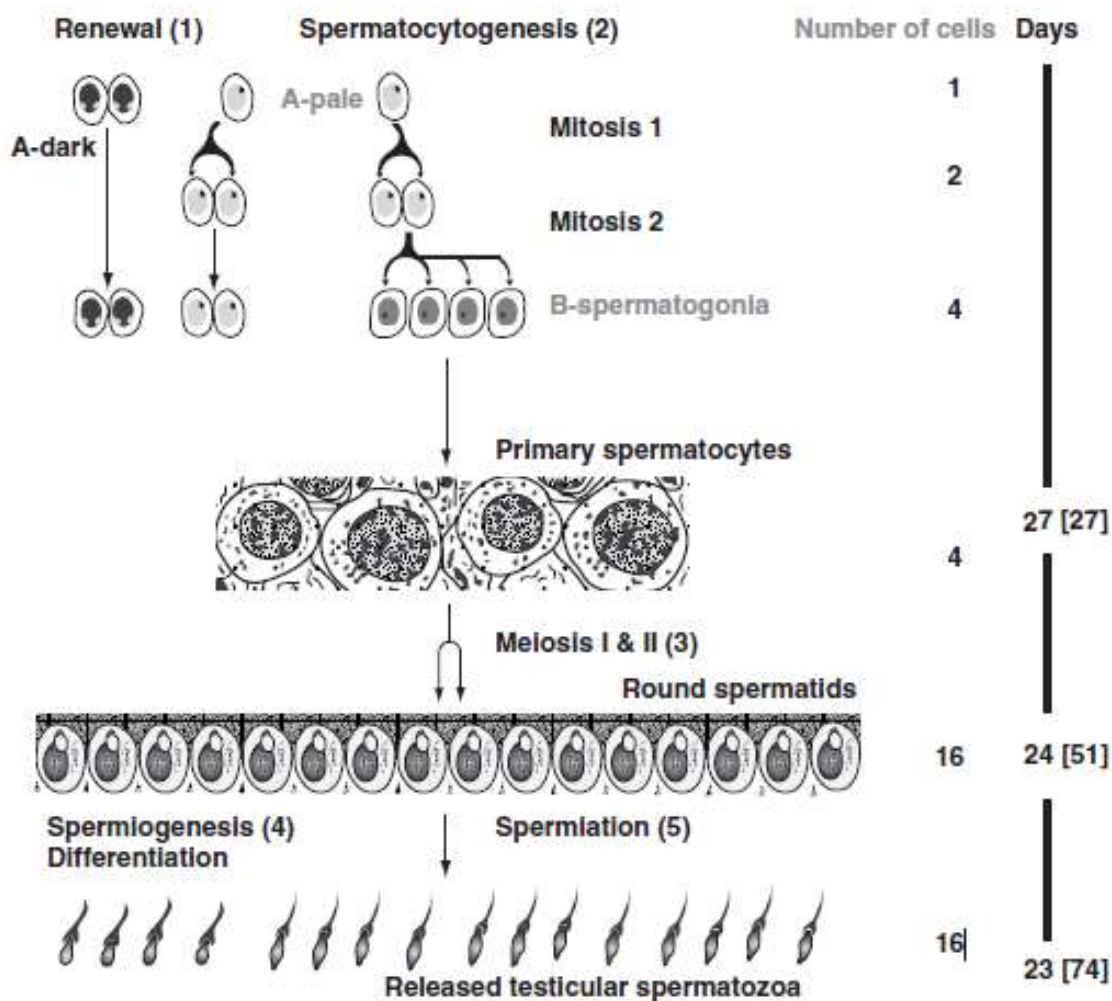


Figure 4. Diagram of human spermatogenesis. (© 2003 Holstein et al; licensee BioMed Central Ltd. <http://www.rbej.com/content/1/1/107>)

The purpose of the next stage, meiosis, is to ensure that every spermatozoon ends up with a unique combination of DNA and a haploid genome were the original 23 pairs of chromosomes are reduced to 23 single copies of DNA. Each one of the four primary spermatocytes undergo two meiotic divisions and the end result is 16 round spermatids. The spermatid is a more or less round cell which contains a nucleus, a Golgi apparatus, a two centrioles and mitochondria.

During the next stage, spermiogenesis, the round spermatids differentiate into testicular spermatozoa which are functional but non-motile. This stage is comprised of 4 different phases

- 1) The Golgi phase when the spermatids begin to develop polarity and the head forms at one end and the Golgi apparatus releases enzymes and packages them with the membranes forming a vesicle that will become the acrosome. At the opposite end a thick mid-piece develops and this is where the mitochondria aggregate, and the axoneme begins to form in the distal centriole leading to the tail. During this phase the DNA also gets packaged and becomes highly condensed. Most somatic histones are exchanged for basic nuclear proteins that later get replaced by cysteine rich protamines and the nuclei accumulate zinc. This occurs during spermatid elongation and results in a tightly packed semi crystalline chromatin.
- 2) Cap/Acrosome phase during which the acrosomal cap is formed from the Golgi apparatus surrounding the condensed nucleus.
3. Formation of the tail where the tail develops, and the head finds its final form. A temporary structure called the manchette, made up of tubules wraps around the nucleus and is believed to assist in the elongation and modeling of the head structure. During this phase the orientation of the spermatozoa results in the tails pointing towards the center of lumen and the head towards the epithelium.
- 4) Maturation phase during which any remaining cytoplasm is removed by the surrounding Sertoli cells by phagocytosis.

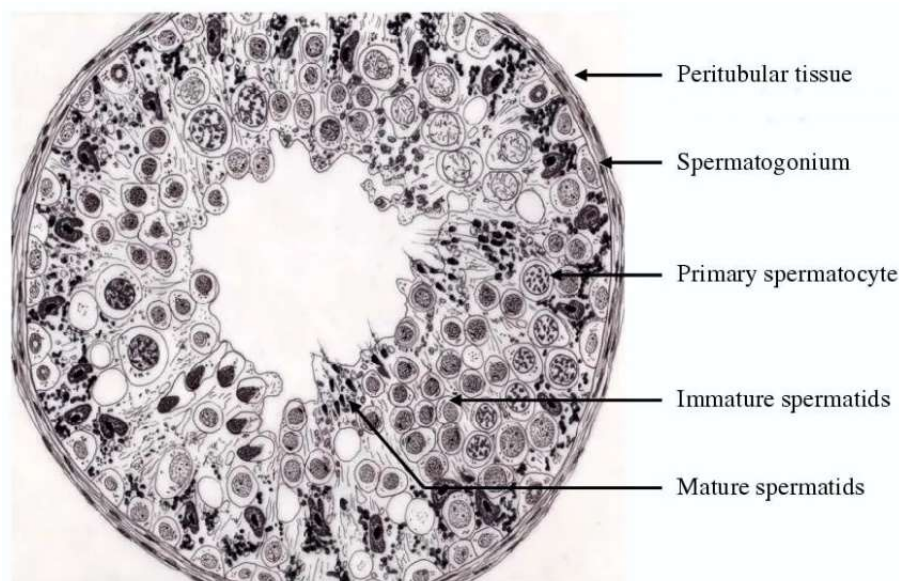


Figure 5. Cross section of a seminiferous tubule (© Holstein et al; licensee BioMed Central Ltd. 2003. <http://www.rbej.com/content/1/1/107>)

At the end of spermiogenesis, spermiation takes place; the cell bridges between the now mature spermatozoa are broken and the spermatozoa are disconnected from the Sertoli cells into the lumen of the seminiferous tubules. The Sertoli cells secrete a fluid called testicular fluid in which the mature but immotile spermatozoa are transported to the epididymis. The transport is achieved by peristaltic contractions. It is during their time in the epididymis that the spermatozoa acquire their ability to be motility. The motility is necessary for transport in the

female reproductive tract, however in the remainder of the male reproductive tract the transport is achieved by muscular contraction only.

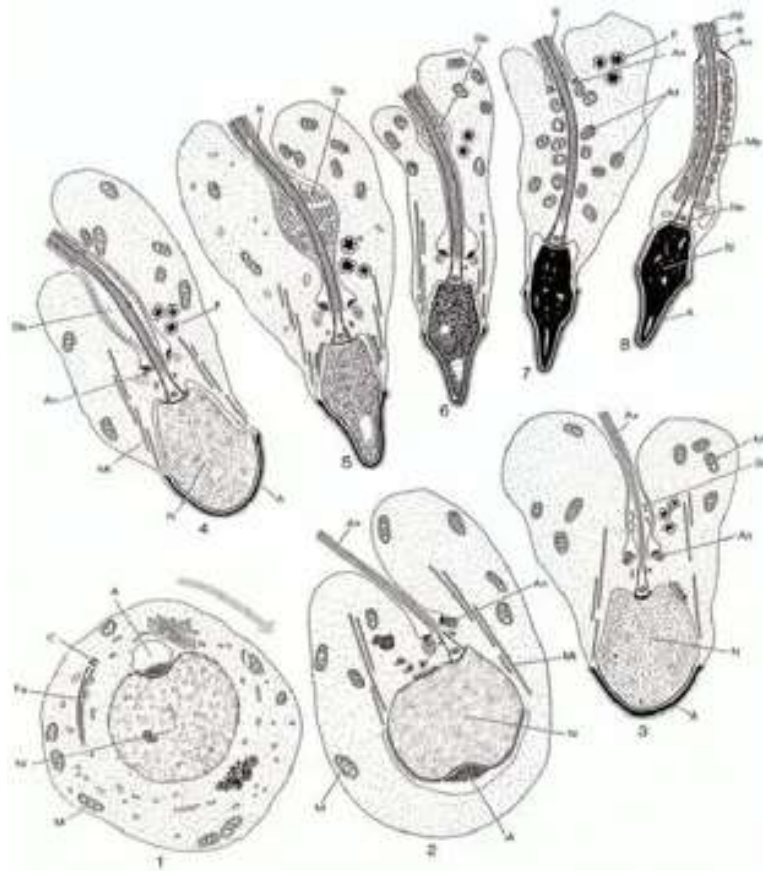


Figure 6. Steps in the differentiation of the spermatid: 1. Immature spermatid, nucleus round shaped 2. The acrosome increases in size and the tail reaches the nucleus. 3-7. Formation of the acrosome, nuclear condensation and development of the structures of the tail. 8. Mature spermatid (© Holstein et al; licensee BioMed Central Ltd. 2003). <http://www.rbej.com/content/1/1/107>)

Ejaculation

Ejaculation is the expulsion of spermatozoa and fluid out of urethra. It is most commonly accompanied by sexual arousal, erection and orgasm. Even prior to the onset of ejaculation there is a mucus-rich fluid secreted from the bulbourethral gland which role is said to help lubricate the urethra. When a sufficient level of stimulation has been reached emission occurs and is controlled by the sympathetic nervous system. A short burst of activity in the nervous system leads to secretion of noradrenalin which in turn stimulates α_1 -receptors on the smooth muscle cells in the cauda epididymis, the vas deferens, the prostatic ducts, the seminal vesicles and the neck of the urinary bladder. This leads to a rapid transport of spermatozoa and fluid to the prostatic part of the urethra. In the prostatic gland there are 20-30 glandular compartments that now empty their secretions, in which the sperm primarily become suspended. The specific sequence of ejaculation can be explained by the structural differences in the anatomy of the glands themselves, the amount of smooth muscles and density of innervation, the actual

diameter of the lumen and the difference in the viscosities of the fluids. The emptying goes in order vas deferens, prostatic acini and finally the wide seminal vesicles.

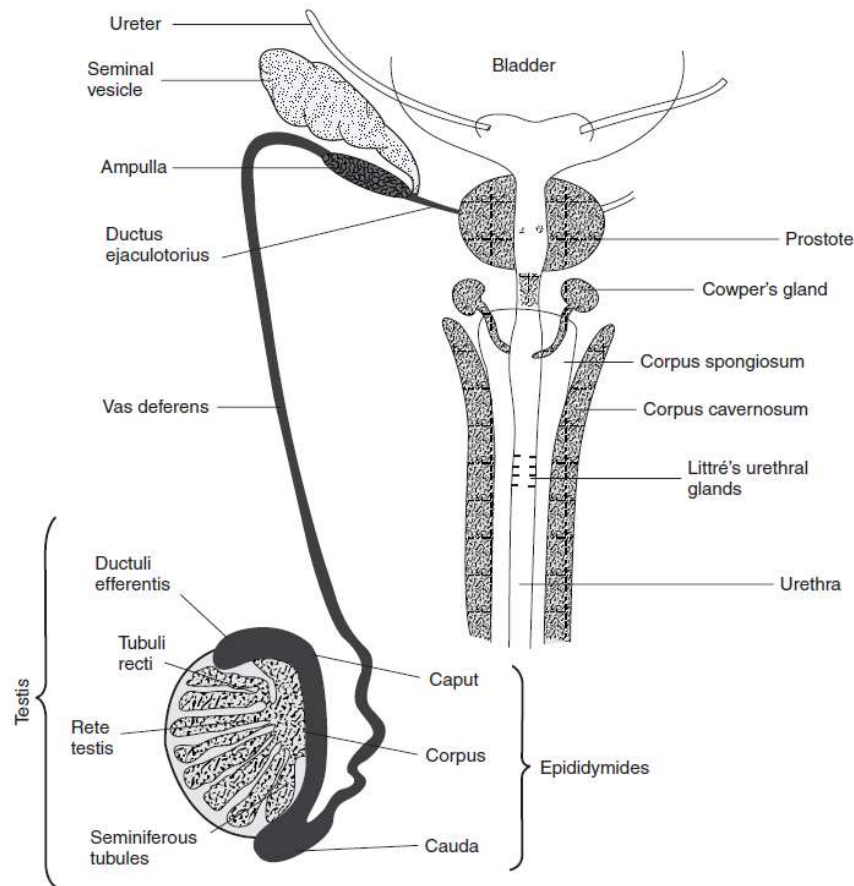


Figure 7. Anatomy of the genital tract in man (Request pending with Cambridge University Press, A Practical Guide to Basic Laboratory Andrology, 9780511723308)

The muscles are controlled by the sympathetic nervous system and the spinal reflex via the pudendal nerve at the level of the spinal nerves S1-4. This also stimulates a simultaneous contraction of the smooth muscles of the bladder neck that normally prevent semen to reach into the bladder. Dilation of the urethra causes a reflex that lead to rhythmic contractions of the bulbourethral, pubococcygeus and ischiocarvernosus muscles. The muscular contractions increase the pressure around and thus in the urethra and cause the semen to be expelled outwards. The semen is expelled in different fractions; the first fraction is the so-called sperm rich fraction which contains most of the spermatozoa suspended in the prostatic fluid. Many studies have looked closer at the different fractions of the seminal fluid and the first fraction contains high concentrations of zinc that is used as a biomarker for prostatic fluid as well as prostate-specific antigens (PSA). PSA is a glycoprotein serine protease enzyme and is associated with degradation of seminal vesicular proteins. During intercourse the first fraction, spermatozoa in prostatic fluid is deposited onto the isotonic cervical mucus at the cervix. Meanwhile, the later fractions gradually contain higher levels of fructose that is used as a biomarker for fluid from the seminal vesicles. There is also a gradual increase in peptides,

proteins and prostaglandins in the later fractions (Huggins and Johnson 1933; Lundquist 1949; Marmar JL 1980; Mann and Lutwak-Mann 1981). When human semen is collected in the split fraction procedure, different containers are used for the different fractions; the first fraction does not form a gel while the last fractions do. When the different fractions are mixed as happens during a normal collection of semen in one container, the gel in the later/seminal vesicular fractions will be dissolved by the proteolytic enzymes present in the first/prostatic fraction and the sample gets liquified.

Capacitation

During sperm passage to and storage in the cauda epididymis sperm conservation factors are added. In order to become fertilizable, it is believed that these factors have to be removed. This process by which the sperm become ready for fertilization is called capacitation. At the onset of capacitation there is an influx of Ca^{2+} which will cause an increase in motility more known as hyperactivation. The influx of Ca^{2+} happens due to the secretion of sterol-binding compounds in the uterus. A decrease in the concentration of sterols causes a more fluid membrane which is more permeable to Ca^{2+} . When capacitation has occurred, the spermatozoa need to get activated and undergo the acrosome reaction.

Acrosome reaction

The acrosomal reaction normally takes place in the ampulla of the fallopian tube (site of fertilization) when the sperm penetrates the cumulus cell mass of the ovum and reaches the zona pellucida. A few events precede the actual acrosome reaction. The sperm cell acquires a "hyperactive motility pattern" by which its flagellum produces vigorous whip-like movements that propel the sperm from the crypts in the isthmus where sperm are stored through the gelatinous content of the fallopian tube towards the ampulla and the outer surface of the sperm then bind with glycoproteins on the zona pellucida of the ovum of that specific species.

The 50 layers of cumulus cells are embedded in a gel-like substance made primarily of hyaluronic acid and developed in the ovary with the egg and support it as it grows. During penetration of the cumulus mass the acrosomal reaction releases hyaluronidase from the acrosome that might facilitate the penetration through the hyaluronic acid surrounding the cumulus cells. However, sperm progressive motility seems to be most important since also other moving organisms, such as spirochetes easily passes through (Talbot 1982). After binding to the zona pellucida the actual acrosome reaction finalises and expose acrosin attached to the inner membrane of the sperm

Acrosin might facilitate penetration of the zona pellucida by digesting zona pellucida glycoproteins (ZP1-3). The sperm membrane in the equatorial segment bind to and fuses with the egg cell membrane and the "nude" sperm sinks into the egg cytoplasm.

In the mouse it has been demonstrated that ZP3, one of the proteins that make up the zona pellucida, binds to a partner molecule (to the $\beta 1,4$ -galactosyl transferase receptors) on the sperm (Jovine 2011). This lock-and-key type mechanism is species-specific and prevents the sperm and egg of different species from fusing. The fused secondary oocyte undergoes a cortical reaction which causes the release of Ca^{2+} that induces hyperpolarization of the oocyte

membrane that can hinder later arriving spermatozoa to bind and fuse with the secondary oocyte. This mechanism is a way to block polyspermy from occurring.

At fusion, the process of egg-activation occurs, and the secondary oocyte undergoes its second meiotic division. The two haploid nuclei from sperm and oocyte, respectively, form the two pronuclei (paternal and maternal) that fuse to form one nucleus after cell division.

Thus, enclosed by the zona pellucida now resides one zygote. One should bear in mind that a certain percentage of sperm cells will undergo a spontaneous acrosome reaction without the presence of an ovum. Those cells are not able to fertilise the egg, even if they do reach it later. Other spermatozoa will spontaneously shed their acrosome during the process of necrosis and appear as acrosome reacted in the light microscope.

Immediately at fusion the sperm chromatin starts to decondense from the distal end of the head. The sperm chromatin gets enclosed by a new membrane and appears as the male pronucleus. Within the pronucleus the protamine's are exchanged for somatic histones and condense into 23 paternal interphases like chromosomes. DNA repair starts and damage to the DNA acquired during sperm transport (epididymis, ejaculation, sperm preparation, female genital tract) will be repaired to various degrees. The circular sperm mitochondrial DNA is labelled with ubiquitin and is thus bound to be destroyed when sperm enter into the ooplasm. Mitochondrial DNA is inherited through the maternal line although exceptions have been discussed.

1.3 OSMOLALITY

Osmolality is defined as the concentration of osmotically active particles in a solution. It is only related to the number of particles and not to their molecular weight, size, shape or charge. The term **osmolarity** refers to the number of particles per **liter** of solution meanwhile the term **osmolality** refers to the number of particles per **kilogram** of solution. When it comes to aqueous solutions such as the different fluids in the human body or media designed to support cells in culture the difference between the two terms is very small. In clinical medicine, the term osmolality is used most often; in this thesis the term osmolality is used, and it has been measured in milliosmoles per kilogram (mOsm/kg).

1.4 HISTORIC ROLE OF OSMOLALITY IN REPRODUCTION

Sperm tail coiling in response to hypoosmotic challenge

As early as 1856 Kölliker reported that spermatozoa suddenly exposed to water underwent an immediate coiling of their tails (Kölliker 1856). Tail coiling after a hypotonic challenge has been shown to be permanent and can be explained by water uptake, swelling of the sperm, and tail coiling (Drevius 1963 and Drevius & Eriksson 1966). Since the sperm membrane surface area does not increase, the swelling cell becomes more spherical and shortened, forcing the tail structure to coil and fold inside the cell membrane (Drevius & Eriksson, 1966). Hypoosmotic swelling also results in lower sperm density and an increased vulnerability to membrane rupture during centrifugation. This is especially true for the initially most swollen spermatozoa (Drevius & Eriksson, 1966). The principle of hypoosmotic swelling is made use of in the HOS-

test to distinguish between live, swelling spermatozoa and dead, non-swelling spermatozoa (Jeyendran et al. 1992).

Osmolality of human semen

As mentioned previously, as early as 1856 it was reported that spermatozoa suddenly exposed to water will coil their tails (Köl liker 1956) A century later it was reported that the osmolality of fresh human semen measured by the method of freeze-point depression was 296 mOsm/kg (Keitel et al 1956). Shortly thereafter it was reported that there was a change in freeze-point depression of seminal plasma stored for 4 h *in vitro* (Rotschild 1960), corresponding to an increase from 306 to 376 mOsm/kg. The question of how semen osmolality changes after ejaculation and its relevance for sperm selection, sperm cryopreservation and consequences for sperm motility and fertilizing capacity remained to answered.

Human seminal plasma examined exactly 60 min after ejaculation was reported to have a mean of 366 (SD 16) mOsm/kg among 29 men with normozoospermic semen samples (Velazquez et al 1977).

The osmolality in 104 fresh human ejaculates analyzed within 1h after ejaculation was found to have a similar mean of 377 mOsm/kg with a range between 300 and 380 mOsm/kg (Makler et al 1981). The variation in osmolality was discussed to be the result of variation in hypertonicity of the secretions from the prostate and the seminal vesicles. Differences in osmolalities were thus reported and no obvious explanations found. In another study, seminal plasma from 78 normospermic men were to have a mean osmolality of 369 ± 33 mOsm/kg (Polak and Daunter 1984).

2 AIMS

In this thesis project we have investigated the changes in semen osmolality occurring in the samples after ejaculation as if collected for ART and how it changes over time. Following that some of the issues addressed are how the change in osmolality can affect the essential functions of spermatozoa. Also, if these affects are significant enough to change the outcome of procedures used in ART. The results are summarized in a series of manuscripts. Manuscript I and II are published meanwhile manuscript III has been submitted and awaits revision. Manuscript IV is in manuscript form and will be submitted.

Study I

- To assess osmolality in the sperm rich, first ejaculated fraction of the ejaculate
- To assess how different individual semen samples change in osmolality after liquefaction
- To assess how different incubation temperature affects the change in osmolality

Study II

- To investigate possible factors influencing the increment of semen osmolality to uncover various intrinsic properties of the human ejaculate
- To investigate the role of the enzymatic degradation for increase in osmolality
- To investigate how dilution of the ejaculate would affect the increase in osmolality

Study III

- To assess how sperm motility is affected by hypotonic challenge for the sperm
- To assess how hypoosmotic challenge affects tail morphology

Study IV

- To assess how the storage temperature after ejaculation affects the yield after density gradient.
- To assess how ejaculate osmolality and yield after gradient centrifugation is changed with time after ejaculation
- To investigate whether dilution of the sample at ejaculation affects the yield after density gradient centrifugation and sperm DFI%
- To investigate whether sperm DFI% changes with increased time after ejaculation

3 MATERIALS AND METHODS

3.1 AT A GLANCE

	MATERIALS	METHODS	STATISTICS
I	<ul style="list-style-type: none"> -88 ejaculates from men referred for semen analysis. -6 ejaculate pools from men referred for semen analysis. -5 split ejaculates from healthy donors 	<ul style="list-style-type: none"> -Osmolality was measured by freeze-point depression. -A regular laboratory centrifuge with a swing-out rotor was used. 	<ul style="list-style-type: none"> -Wilcoxon matched pairs signed rank test. -T test -One-way ANOVA -Friedman test -Pearson correlation -A p-value of <0.05 was considered significant
II	<ul style="list-style-type: none"> -208 ejaculates from men referred for semen analysis Whereof 48 azoospermic samples and 40 samples from men post vasectomy -1 ejaculate pool from a man referred for semen analysis 	<ul style="list-style-type: none"> -Osmolality was measured by freeze-point depression. -Zinc was measured by a colorimetric assay. -Fructose was measured by an acid indole assay -Neutral α-Glucosidase activity was determined using an assay 	<ul style="list-style-type: none"> -Wilcoxon matched pairs signed rank test. -T test -One-way ANOVA -Friedman test -Pearson correlation -A p-value of <0.05 was considered significant
III	<ul style="list-style-type: none"> -22 ejaculates from healthy volunteers 	<ul style="list-style-type: none"> -Osmolality was measured by freeze-point depression. -CASA was used for motility analysis. -Double layered density gradients for selection of sperm 	<ul style="list-style-type: none"> -Wilcoxon matched pairs signed rank test -T test -One-way ANOVA -Pearson correlation -A p-value of <0.05 was considered significant
IV	<ul style="list-style-type: none"> -30 ejaculates from healthy volunteers 	<ul style="list-style-type: none"> -Osmolality was measured by freeze-point depression. -Double layered density gradients for selection of sperm. -DFI% and %HDS was measured on acridine orange stained samples by flow cytometry. 	<ul style="list-style-type: none"> -Wilcoxon matched pairs signed rank test -T test -One-way ANOVA -Pearson correlation -A p-value of <0.05 was considered significant

3.2 RECRUITMENT

In study I, the participants were either patients referred to the clinic for semen analysis due to unspecific couple infertility or healthy donors. In study II the participants were a mix of patients referred to the clinic for semen analysis due to unspecific couple infertility, men referred for semen analysis post-vasectomy and healthy donors. In study III and IV, the participants were all healthy donors. Any samples that had a volume below 1 mL, as well as if the concentration was too low, were excluded. Since the purpose of the study was to experimentally examine how spermatozoa react to certain changes in their environment and not in correlation with any infertility problems, the recruitment requirements was to have a large enough volume and enough motile spermatozoa to perform the analyses and experiments properly. All participants, volunteers and patients, had previously been informed of the experiments and gave their written consent to participate. All participants were informed to adhere to 2-4 days of abstinence prior to each collection. If the samples were collected at home and during the colder months of the year, the donors were told to keep the sample close to the body in order to keep the temperature stable.

3.3 METHODS

Ethical Approval

All the procedures used in this thesis was done in accordance with the Ethical Approval (DNR 2015/2326-31) that has been issued for the clinic and the laboratory. All four studies were conducted as experimental studies.

In study I and II the samples were collected by masturbation at the clinic. The collection containers and all the paperwork for each person were assigned a specific laboratory number. All samples were analyzed according to WHO (World Health Organization, 2010) and recommendations by the Special Interest Group in Andrology of the European Society of Human Reproduction and Embryology (Barratt, Björndahl, Menkveld, & Mortimer, 2011).

In study III and IV the samples were collected mainly at the home of the donors however in certain experiments where the time between ejaculation and the start of analysis were of importance the samples were collected at the laboratory.

Measuring Osmolality

All the osmolality measurements in this project were performed using an Automatic Osmometer 13 (Löser, Svenska Labex AB) using the principle of freeze-point depression. Standard calibrations were performed once a day prior to starting the experiments with deionized H₂O (0 mOsm/kg) and once every month with a 300 mOsm/kg standard solution. When measuring a sample, 100 µL of the sample solution was placed in a 1.5 mL micro centrifuge tube (Sarstedt) and placed on the measuring head and the measurement was

performed. Every measurement was done in duplicate and an average of the values would be used and if any discrepancy was noted a new aliquot of the sample would be analyzed.

Preparation of media

The media used for diluting, washing and selecting spermatozoa and ejaculates were commercially available standard media (provided by Nidacon International AB, Mölndal, www.nidacon.com). When the desired osmolality was not that of the standard commercially available solution the right osmolality was achieved by adjusting the glucose, NaCl and KCl levels in the media by calculating the amount needed and then fine adjusting the final osmolality while blending the media. All the other constituents were kept to the standard proportions, such as the silinized silica in the density gradients. All these adjustments were made under the same conditions as the standard media (during production in the cleanroom). See table 1. below for more details. The only exception was the Earle's Balanced Salt solution used in study I which was made at hand in the laboratory according to a standard recipe.

Medium type	Osmolalities (mOsm/kg)	Method of preparation
Density Gradients	300	Commercially available ready-to-use Nidacon PureSperm® 40 and 80 or PureSperm®100 and PureSperm Buffer mixed to make a 40 and 80 (the same composition as the ready-to-use solutions)
Density Gradients	400	Custom made to the right osmolality. Silinized silica maintained at proportions used in regular PureSperm® 40 and 80 density gradient media while other ingredients were increased proportionally to obtain the osmolality of 400 mOsm/kg
Density Gradients	290, 310, 330, 350	Commercially available Nidacon PureSperm® 40 and 80 was used and a stock solution with NaCl, KCl and Glucose was used to increase the osmolality to the intended levels. For the 290mOsm/kg gradient the PureSperm® was diluted with a mix of silinized silica and ddH ₂ O
Washing medium	50, 100, 290, 300, 310, 330, 350, 400, 500	Commercially available PureSperm® Wash to which a stock solution made with NaCl, KCl and Glucose was added to obtain the intended osmolalities. For the 100 and 50 mOsm/kg the standard PureSperm® Wash was diluted with ddH ₂ O and then adding extra hSA to a content of 2.4% to maintain the original protein level
Buffered salt solution	300	Commercially available PureSperm Buffer which is an isotonic HEPES buffered salt solution that does not contain HSA (human serum albumin)

Table 1. Preparation of media with specific osmolalities. (From Study III, Table 2)

Storage of semen samples and temperature

In several of the experiments the semen samples were kept at 37°C and this was done in a temperature-controlled heating cabinet. When the samples were kept at room temperature (20-22°C) this meant they were kept on the benchtop in the temperature-controlled laboratory. In study I part of the samples were exposed to 100°C boiling water for 5 min. This was done in a standard temperature-controlled laboratory water bath. The samples were cooled, and osmolality was measured. Also, in study I samples were kept at -18 to -20°C and this was accomplished by placing them in a laboratory deep freezer. In addition, samples were kept at temperatures of 4-8°C and this was accomplished by placing them in a standard laboratory refrigerator.

Biochemistry

Zinc was measured using a colorimetric assay (Wako Pure Chemicals GmbH) (Johnsen & Eliasson, 1987). Fructose was determined using an acid indole assay (Karvonen & Malm, 1955). α -Glucosidase was assessed using an assay for the determination of neutral α -Glucosidase activity by measurement of the conversion of p-nitrophenol-glucopyranoside into p-nitrophenol which, together with Na_2CO_3 , forms a complex that absorbs light of a certain wavelength making quantitation of the enzyme activity possible (Cooper et al. 1990). The results for concentration of zinc and fructose and neutral α -Glucosidase activity were obtained from routine extended semen analysis results. Zinc and fructose concentrations and α -glucosidase activity were measured in sperm-free seminal fluid (centrifugation 3,000 g, 20 min). Total amounts of zinc ≥ 2.5 μmol /ejaculate (zinc concentration ≥ 1.2 mmol/ml), fructose ≥ 13 μmol /ejaculate and α -Glucosidase activity ≥ 20 mIU/ejaculate are considered indicative of normal prostatic, seminal vesicular and epididymal secretory functions, respectively (Björndahl et al., 2010).

Enzymatic Activity

In study II one aim was to examine if the addition of enzyme inhibitors could stop the increment in osmolality since it has been previously proposed that there is an enzymatic breakdown of large molecules happening in the samples (Kubicek et al. 1959; Mann 1964; Quinlivan, 1972). Three stock solutions of inhibitors were made: (a) nuclease- and metallo-protease inhibitor EDTA (0.2 M Na_2 -EDTA of neutral pH), (b) acid phosphatase inhibitor tartrate (1 M Na_2 -tartrate) and (c) protease inhibitor mixture cOmplete™ (EDTA-free Protease Inhibitor Cocktail Roche; 1 tablet dissolved in 200 μl water 50 \times). 25 μl of each stock solution, was added to 2.5 ml seminal fluid to give final concentrations of EDTA 2 mM; tartrate 10 mM and cOmplete 0.5 \times . Aliquots of seminal fluid, frozen at 3–4 hours after ejaculation, were thawed, pooled and subdivided into two parts of 2.5 ml each. To one part, 75 μl water was added (control), and to the other part, 25 μl of each of the three inhibitor solutions was added. Samples were then thoroughly mixed.

Double Layer Density Gradient centrifugation

A common method for selection of spermatozoa prior to ART is double layered density gradient centrifugation (300 x g for 20 min). The separation is based on the buoyant density of the sperm and is achieved when spermatozoa with higher density goes through two discontinuous layers of colloidal silinized silica and collects in a pellet at the bottom of the centrifuge tube. The seminal plasma and other cells and debris as well as sperm with a lower density remains in the supernatant. As it is, the pellet contains predominantly high quality and motile sperm. The supernatant is removed with a Pasteur pipette and discarded. The pellet is then retrieved and transferred to another conical centrifuge tube containing washing medium (PureSperm® Wash). This step will ensure the spermatozoa are clean and it will also eliminate any silinized silica that remains in the pellet from the previous step. The washing medium containing the sperm and will then be centrifuged (500 × g for 10 min). The supernatant is removed and discarded, and it is the sperm in this pellet that will be used in the experiment.

See how the different solutions used for the density gradient was prepared in Table 1, “Preparation of media”. The protocol used for the density gradient in study III and IV can be seen here in Table 2 below.

Protocol for density gradient centrifugation	
1.	1 mL of 80 (bottom layer) is placed in a conical centrifuge tube
2.	1 mL of 40 (top layer) is carefully layered on top of the 80
3.	A maximum of 500 µL of semen sample is carefully layered on top of the gradient.
4.	Tubes are placed in a centrifuge at 300 x g for 20 min.
5.	The tubes are removed carefully, and the supernatant is aspirated and discarded, leaving only the pellet and 4-6 mm of liquid above it.
6.	The pellet is then picked up and resuspended in 4mL of washing medium.
7.	The sperm and washing medium are then centrifuged at 500 x g for 10min
8.	The supernatant is removed, leaving the pellet and an estimated amount of media so that the concentration will be about 15-25 mill/mL. The estimation is depending on the size of the pellet and how tightly packed it is. The amount is recorded.
9.	The sample was now ready to be analyzed for concentration, motility or fixed for tail coiling analysis.

Table 2. Protocol of double layered density gradient. (From Study IV, Table 1)

Analysis of motility by Computer Assisted Sperm Analysis

Motility assessments were done by Computer Assisted Sperm Analysis (CASA) with the Hamilton Thorne (www.hamiltonthorne.com) IVOS version 12.3D (Build 002) on gradient selected and washed spermatozoa using the pre-programmed setting “washed human”. The details of this setting are presented in Table 3.

Image capture	Frames /sec 60Hz	No of frames 30
Cell detection	Min contrast 80	Min cell size 3
Defaults (if < 5 motile cells)	Cell size 6 pix	Cell intensity 160
Progressive cells	VAP 25.0 $\mu\text{m/s}$	STR 80%
Slow cells (included in motile)	VAP cut-off 6.0 $\mu\text{m/s}$	VSL cut-off 5 $\mu\text{m/s}$

Table 3: Settings for the program “Washed Human Semen” in Hamilton Thorn IVOS. (From Study III, Table 1).

For analysis 5 μL of a sperm suspension was placed in duplicate in a double 20 μm chamber (Leja) and then analyzed. The concentration and total number of spermatozoa, motile spermatozoa, and progressively motile spermatozoa ($\geq 25 \mu\text{m/s}$) were recorded in sperm populations selected by density gradient centrifugation. Also, sperm motility patterns (kinematics: VAP (average path velocity), VCL (curvilinear velocity), and VSL (straight line velocity)) were recorded for the sperm populations. The definitions of these motility patterns can be seen below.

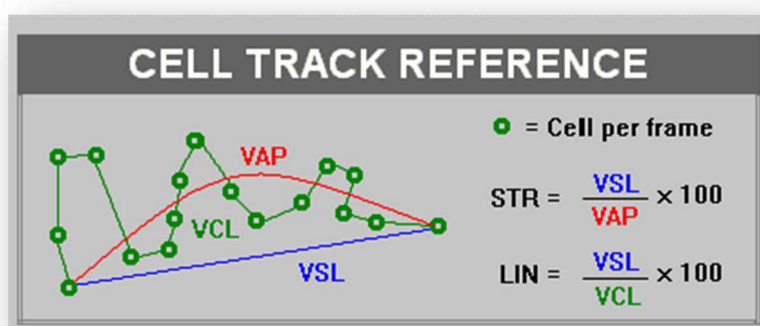


Figure 8. Schematic explanation of Curvilinear Velocity (VCL), Average Path Velocity (VAP) and Straight Line Velocity (VSL). Image courtesy Hamilton Thorne, Inc., Beverly MA USA, www.hamiltonthorne.com

Tail coiling

During the initial analysis of the motility patterns it was noted that the sperm had a very specific pattern best described by them having something heavy attached to the end of their tails. This resulted in that the sperm were fixed with a modified Karnovsky's solution (2% glutaraldehyde,

2% paraformaldehyde, 0.1 M sodium cacodylate buffer, pH 7.4) after which they were analyzed under phase contrast. It was apparent that many of the tails were affected and some more than others. Therefore, a categorization scheme for analysis was developed, and studies were designed to investigate further how changes in osmolality affected the tail tip coiling and tail folding. See category scheme can be seen in figure 9 below.

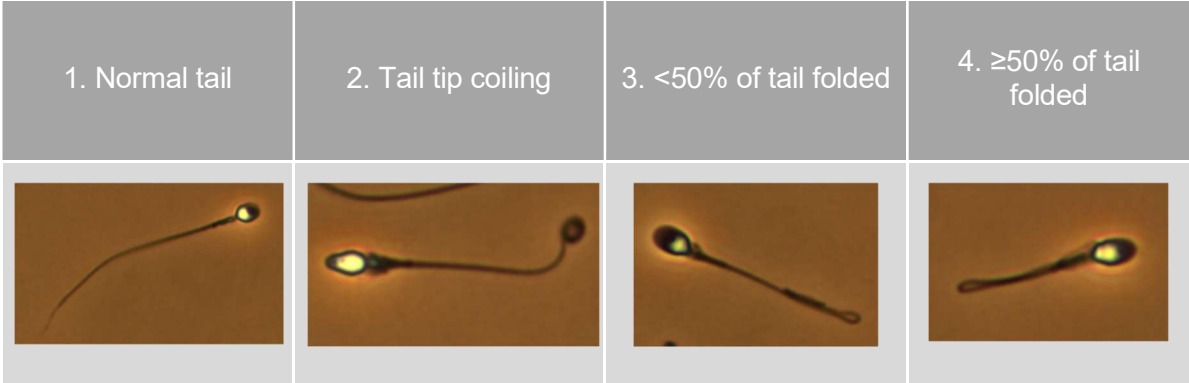


Figure 9. Categorization scheme for analyzing the effect of osmolality changes on tail tip coiling and folding. (From Study III, Fig. 2)

Defragmentation Index (DFI%) and High DNA-stainable sperm (%HDS)

Samples that were analyzed for DFI and HDS were frozen in 100µL aliquots put into 1 mL centrifuge tubes and then placing them in the deep freezer. The samples were thawed just prior to analysis. Thawing of samples was done by adding the TNE buffer (150 mM NaCl, 10 mM Tris, 1 mM EDTA, pH=7.4) to adjust to 2×10^6 /ml and mixing thoroughly. The sample was then placed on ice immediately after thawing. Measurement of all samples was carried out within 1 hour after thawing on BD FACSTrack as previously described by (Evenson & Jost, 2001), with minor modification:

An aliquot of 100 µL kept on ice was mixed with 200 µL of acid detergent for 30 seconds and then stained by adding 600 µL of 6 µg/mL acridine orange in buffer and measured after 150 seconds. Samples were measured in batches of max 6 samples after equilibration of AO within the flow cytometer and adjusting instrument settings according to the reference sample. All measurements were done in duplicates, a total of 5000 events excluding debris were collected in each run.

Experimental design Study 1

Reproducibility of the method for measuring osmolality was performed by taking 10 replicate measurements of 3 different buffered salt solutions with known osmolalities 300, 350, and 400 mOsm/kg. Measurements of semen was also taken, 35 measurements in repeat.

In order to measure the osmolality of the very first sperm-rich fraction of the ejaculate, samples were collected by masturbation in split fractions. The first, second and third fractions were collected in three separate collection containers (Björndahl & Kvist, 2003). The remaining fractions were collected together in the fourth container. The fractions were kept at 37°C and

the osmolality of each fraction was measured separately. Only the results from the first fractions, where most spermatozoa are expelled (Lundquist, 1949; MacLeod & Hotchkiss, 1942), are presented in the Results section.

In order to study possible changes in the osmolality after liquefaction, the osmolality of 47 different individual semen samples were measured soon after liquefaction and again 3 hours later. Prior to the first measurement and during the 3 hours of storage the samples were kept at 37°C. The incubation time was used as an experimental model and it does not indicate a clinically relevant duration.

In order to examine how temperature affects the osmolality increment in semen, samples were stored in different temperatures. To get enough sample to split into the 4 temperature groups semen samples were pooled and then split into 24 aliquots. Six aliquots were placed in a freezer (-18 to -20°C), six in the refrigerator (4-8°C), six were stored at room temperature and six were stored at 37°C. Two aliquots from each temperature group was measured at 1, 4 and 24 hours.

Sperm free seminal plasma from 6 individual samples, retrieved by centrifugation of whole semen (300 x g for 20 min) and the supernatant was collected, was used to examine if exposure to a temperature of 100°C would stop the osmolality increment. The reason for using 300 x g in this experiment is that the sperm will be removed but not damaged and other particles will remain in suspension. The theory that any enzymatic activity would be denatured by this treatment was the background to this experiment.

Experimental design Study II

In order to evaluate if the concentration of sperm in a sample had an effect on the osmolality measurement, 3 individual semen samples were centrifuged (400 x g for 10 min) and sperm free seminal plasma was removed. The pellet of sperm was resuspended in the remaining seminal fluid. The concentration of the original sample had previously been established (Björndahl et al., 2010; World Health Organization, 2010). By serially diluting the sperm sample with the seminal fluid from the same sample, six different sperm suspensions were made having 100%, 50%, 25%, 12.5%, 6.25% and 0% of the original sperm concentration. Osmolality was then measured in all six suspensions.

To evaluate whether other large particles present in the semen sample influenced the osmolality measurement, these particles were removed by filtration with a 0.2 µm filter (Acrodisc®, Pall). Three samples of whole semen were divided in two parts where one part was filtered and the other acted as a control. Eight samples of sperm free seminal fluid, achieved by centrifuging samples at 3,000 x g for 20 min and then dividing the samples in two parts, one part was filtered and the other acted as a control. Osmolality was measured in all the different samples 2 hours after ejaculation, again 5 hours after ejaculation (stored at 37°C) and again 24 hours after ejaculation.

To examine whether enzymes or particles that may have leaked from the spermatozoa prior to liquefaction had any significant effect on the increment in osmolality seen after liquefaction,

osmolality was measured on samples that did not contain any sperm (azoospermic samples n=48 and samples from vasectomized men n=40) and on samples containing sperm (normozoospermic n=47). Samples were centrifuged (3,000 x g for 10 min) 3–4 hours after ejaculation and the sperm free seminal fluid was then placed in a freezer at -20°C. Osmolality was measured directly after thawing the samples. The reason for using 3,000 x g in this experiment was to ensure that all sperm were removed

To investigate whether the osmolality increment specifically was related to either of the prostatic, the seminal vesicular or to the epididymal secretion osmolality measurements were taken directly after liquefaction and again after 3 hours of storage at 37°C. These results were compared to measurements of the prostatic marker zinc concentration, the seminal vesicular marker fructose concentration and the marker for epididymal secretion α -glucosidase activity.

To evaluate whether enzymatic activity influences the increment in osmolality, seminal fluid was incubated at 37°C for 24 hours with and without a mixture of enzymatic inhibitors (see details of method under section “Enzyme activity”). Osmolality was measured in duplicates directly after adding the enzymatic inhibitors and again after 18 and 24 hours of incubation in a water bath at 37°C.

The final part of the experiments in study II was to examine if diluting the semen samples would influence the increment of osmolality. Two aliquots of 400 μ L from each of 7 individual semen samples were retrieved after complete liquefaction. To one aliquot an equal amount of 400 μ L of Earle’s balanced salt solution, containing 10% of human serum albumin (HSA) was added. The other aliquot remained undiluted. Osmolality was measured in both aliquots directly after dilution and again after 3 hours of storage in 37°C.

Experimental design Study III

In this study the overall goal was to investigate whether the motility patterns of sperm were affected by a decrease in osmolality (hypoosmotic challenge). In order to examine how motility was affected by hyperosmotic or hypoosmotic changes, spermatozoa in 6 individual semen samples were selected by density gradient centrifugation after liquefaction and thorough mixing (see protocol in section “Density Gradient”). Gradients as well as washing medium had an osmolality of 300 mOsm/kg. The final sperm suspension was the mixed 1+3 with another washing medium that had been adjusted to osmolalities of 300 mOsm/kg, 500 mOsm/kg, 100 mOsm/kg, and 50 mOsm/kg. The mixing schedule can be seen below in table 4.

Osmolality of sperm preparation (mOsm/kg)	Osmolality of added medium (mOsm/kg)	Sperm preparation + medium	Final osmolality (mOsm/kg)
300	300	1+3	300
300	500	1+3	450
300	100	1+3	150
300	50	1+3	112.5

Table 4. Mixing schedule for sperm and washing medium to examine hyperosmotic or hypoosmotic challenges following density gradient selection. (From Study III, Table 3)

Directly after mixing, samples were analyzed by CASA. Samples that had been exposed to 150 mOsm/kg (n=5) were also assessed 10 minutes later to evaluate if any recovery of motility had occurred. See the section on CASA analysis for more details on the method used.

In order to imitate an increment in osmolality that would occur during storage, density gradients and washing medium that had an osmolality of 400 mOsm/kg were used in this experiment. Density gradient selection followed by a wash was performed on 8 individual ejaculates. After the last washing step, 95 μ L of the remaining sperm suspension was transferred into each of two 1.5 mL micro centrifuge tubes (Eppendorf) one already containing 55 μ L of washing medium with an osmolality of 400 mOsm/kg (control) and the other containing 55 μ L of medium with an osmolality of 100 mOsm/kg (test) obtaining a final osmolality of 290 mOsm/kg. The sperm motility was analyzed directly by CASA. The motility was analyzed again after 60 min of incubation at room temperature (20-22°C). During the motility assessment it was noted that the tail morphology of the spermatozoa had changed after an hypoosmotic challenge. Therefore, an analysis of the tail tip coiling and folding of the tail was added to the study. A sample of the test group was fixed and later analyzed for tail tip coiling and tail folding.

In a second part of the experiment, 8 ejaculates were used to evaluate how different hypotonic challenges would affect the sperm tails. Four density gradients were used with differing osmolalities 350, 330, 310, and 290 mOsm/kg, the washing medium for the second cleaning step had the same osmolalities. Then, 190 μ L of each washed sperm suspension was mixed with a calculated amount of wash medium with an osmolality of 100 mOsm/kg in order to obtain a final osmolality of 290 mOsm/kg in each suspension. Another 190 μ L of each suspension was mixed with 100 μ L of washing medium with the same osmolality, so no change in osmolality was experienced, this acted as the control. For a design scheme of this experiment see figure 10 below.

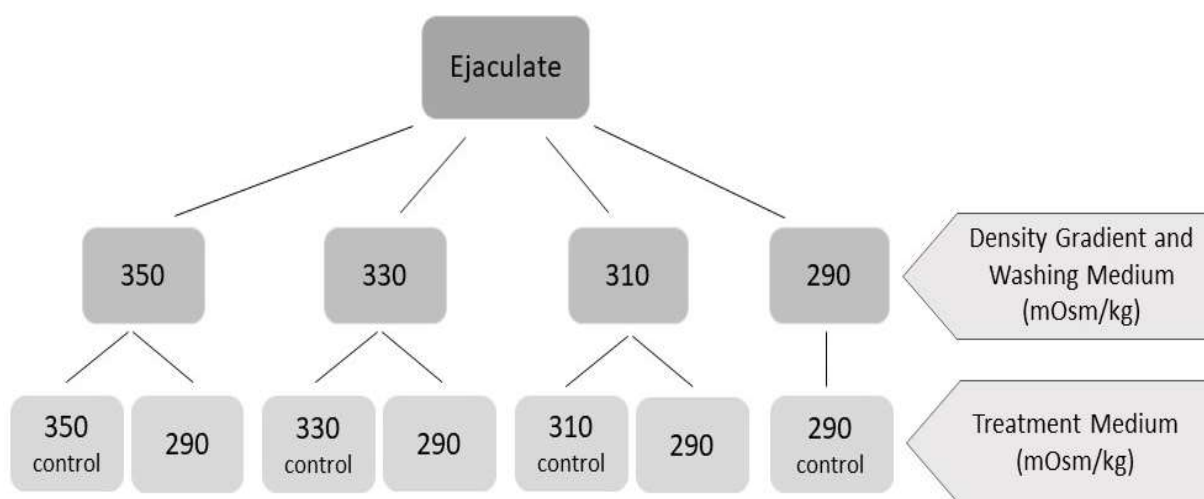


Figure 10. Design scheme on effects on motility caused by decreases in osmolality to 290 mOsm/kg after a density gradient selection of a certain osmolality 290, 310, 330 or 350 mOsm/kg. Each of the density gradients had a control where there would be no decrease in osmolality. (From Study III, Fig 1).

An aliquot of each sample was fixed and analyzed for tail tip coiling and folding according to the categories in Figure 9.

Experimental design study IV

Since it had previously been shown that the most dramatic increase in osmolality in semen samples was seen during storage at 37°C this approach was used to achieve a high osmolality. In total 7 individual semen samples were used, and an initial osmolality was measured prior to placing the samples in the heating cabinet. After 60 min and again after 180 min in 37°C the samples were measured for osmolality and an aliquot was processed on a double layered density gradient. The osmolality and the yield after the density gradient centrifugation before and after 3 hours incubation was compared.

In a control experiment 7 individual ejaculates were stored at room temperature and osmolality was measured and aliquots of 100 µL were retrieved and placed in the freezer (DFI and HDS analysis) soon after liquefaction and again 2 hours later.

In another experiment it was examined whether diluting the samples as soon as possible after ejaculation and storing the samples at room temperature (20-22°C) would give a better yield after a density gradient. A total of 8 individual semen samples were used and they were mixed thoroughly and divided as soon as possible after ejaculation. One part of the ejaculates was diluted with a buffered salt solution (290 mOsm/kg) and the other part was left undiluted. The samples were measured for osmolality and an aliquot of 100 µL was placed in freezer for DFI and HDS analysis, directly after dilution (time 0) and again after 2 hours (time 2). In addition, a double layered density gradient was performed on all samples at time 0 and again 2 hours later.

In order to examine whether the time between ejaculation and liquefaction had a significant effect on the osmolality increment and on the yield after a density gradient, the donors were

asked to ejaculate into a cup already containing 5 mL of buffered salt solution (290 mOsm/kg). The volume of the sample was determined by weight and if the volume was greater than 5 mL an additional 5 mL of buffered salt solution would be added. Therefore, the dilution was always at least 1+1. These samples were measured for osmolality directly and again after 2 hours of storage at room temperature. A double layered density gradient was also performed at the two time points.

4 RESULTS

4.1 RELIABILITY AND REPRODUCIBILITY OF OSMOLALITY ASSESSMENTS

Method variability

Replicate measurements (10 of each) of three different buffered salt solutions with known osmolalities of 300, 350, and 400 mOsm/kg showed a CV of 0.2% and the overall deviation from the expected result was 1 mOsm/kg (Study I).

In 35 ejaculates duplicate measurements were compared: the average for the first series was 383.5 mOsm/kg (range 284-544) and for the repeated second series was 384.3 mOsm/kg (284-544) (Study I).

Presence of spermatozoa and compounds originating from spermatozoa

To investigate the possible influence of presence of spermatozoa to the measured osmolality, three individual ejaculates were examined where the concentration of spermatozoa was serially diluted with sperm-free seminal fluid from the same ejaculate. The concentration of spermatozoa in a semen sample thus did not affect the measurement of osmolality (Study II).

Filtering of whole semen or seminal plasma through a 0.2 μm filter did not affect the measurement of osmolality. In unfiltered whole semen the mean osmolality was 358 mOsm/kg, and in filtered semen it was 359 mOsm/kg. In unfiltered seminal fluid the mean osmolality was 385 mOsm/kg and in filtered seminal fluid it was 384 mOsm/kg. (Study II). Thus, presence of particles larger than 0.2 μm did neither interfere with the actual measurement of osmolality nor significantly contributed to the observed osmolality.

Factors contributing to increasing osmolality

When factors possibly contributing to an increase in osmolality the presence of spermatozoa was investigated. However, the increase in osmolality was the same in whole semen and sperm-free seminal plasma (Study II). The increase in osmolality was also compared between azoospermic ejaculates, post-vasectomy and whole semen. Samples without sperm had a slightly higher osmolality than samples with sperm (Study II).

Prostatic marker zinc was positively related to the increase in osmolality after storage in vitro: 19% of the inter-sample variation in osmolality covaried with the marker for prostatic secretion. Markers for seminal vesicular and epididymal secretions, respectively, did not show a relation with the increased osmolality upon storage in vitro (Study II).

To investigate if enzymatic activity in the ejaculate could be responsible for the increase in osmolality during storage incubation at different temperatures (-18 to -20°C, 4-8°C, 20-22°C, 37°C, respectively) the development of osmolality during 24 hours of incubation was investigated. At 37°C the increase was 196 mOsm/kg, at room temperature 99.5 mOsm/kg, while incubation in refrigerator resulted in a rise with 35 mOsm/kg, and for aliquots stored in a freezer the increase was only 2 mOsm/kg (Study I).

To investigate the effects of denaturing temperature (100°C) comparison with incubation at 37°C was done. While the body temperature incubation caused an increase of 73 mOsm/kg, exposure to 100°C resulted in an increase of only 14 mOsm/kg that could be due to an increased enzymatic activity while increasing the aliquot temperature to 100°C (Study I).

Addition of a mixture of enzyme inhibitors to seminal fluid reduced the increment in osmolality by 83% for 18 hours of incubation and for 24 hours of incubation the increment was reduced by 75% (study II).

4.2 POST-EJACULATORY INCREASE IN HUMAN SEMEN OSMOLALITY *IN VITRO*

Osmolality within 5 min after ejaculation

When osmolality was measured in the first split-ejaculate fraction of 5 individual ejaculates within 5 min after ejaculation the median was 304 mOsm/kg (range 281-308), i.e. near normal osmolality of body fluids. (Study I)

Osmolality in whole semen *in vitro* after liquefaction

After liquefaction (assessed 25-60 minutes after ejaculate collection) the average osmolality of 47 whole ejaculates had an osmolality of 322 mOsm/kg (range 280-361). The mean osmolality of the same 47 samples after 3 hrs of further storage at 37°C was significantly increased with time stored in 37°C (Study I) (Fig. 11 below, from Study I).

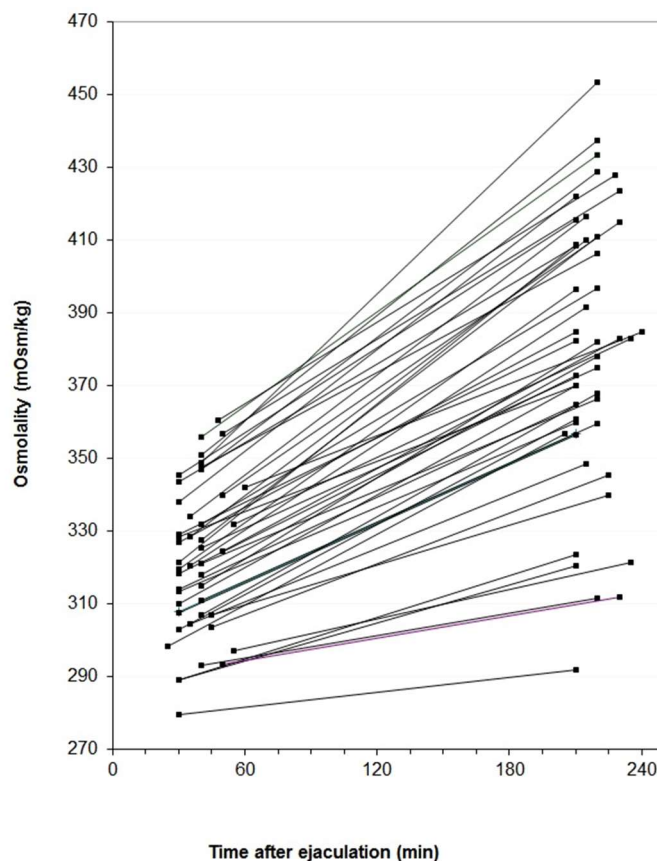


Figure 11. Change in osmolality (mOsm/kg) of 47 whole semen samples incubated for 3 hours at 37°C. First measurement was taken at 25–60 min after ejaculation and second measurement 3 hours later. (From Study I, Fig. 2).

4.3 LABORATORY PROCEDURES TO REDUCE *IN VITRO* INCREASE IN EJACULATE OSMOLALITY OF HUMAN SEMEN

Dilution of semen

Dilution of the ejaculate with a buffered salt solution both reduced the initial osmolality and the increment in osmolality during further storage *in vitro* (Study II, Fig.12 from Study II)

The effect of dilution 1:2 early after ejaculation with a buffered salt solution was assessed after 2 hours of storage at room temperature. Compared to undiluted semen (increase 42 mOsm/kg from a mean of 327 to 369), diluted aliquots only increased by 14 mOsm/kg (from a mean of 308 to 322) (study IV).

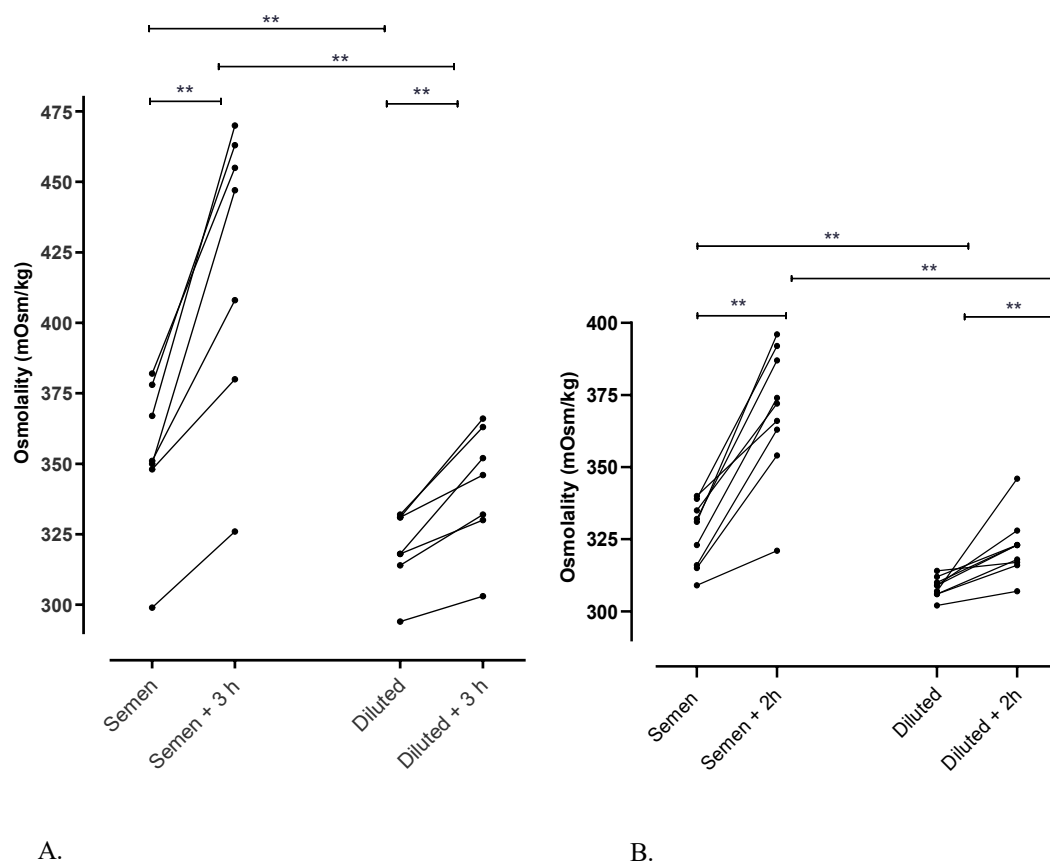


Figure 12. (A) Comparison between the development of osmolality (mOsm/kg) of diluted semen and undiluted semen during 3 hours of storage at 37°C. (From Study II, Fig 5). (B) Comparison between the development of osmolality in diluted semen and undiluted during 2 hours of storage at room temperature (20-22°C). (From Study IV, Fig 6)

4.4 HYPOTONIC CHALLENGE REDUCED HUMAN SPERM MOTILITY THROUGH COILING AND FOLDING OF THE TAIL

Motility after hypertonic and hypotonic challenges

An increase in osmolality from 300 mOsm/kg to 450 mOsm/kg did not reduce the sperm motility as measured by CASA. However, a decrease in osmolality from 300 mOsm/kg to 150 mOsm/kg resulted in a 50% reduction in curvilinear motility. Furthermore, a decrease in osmolality to 112.5 mOsm/kg resulted the sperm being completely immotile. There was no recovery of the reduced curvilinear motility after 10 min. (Study III)

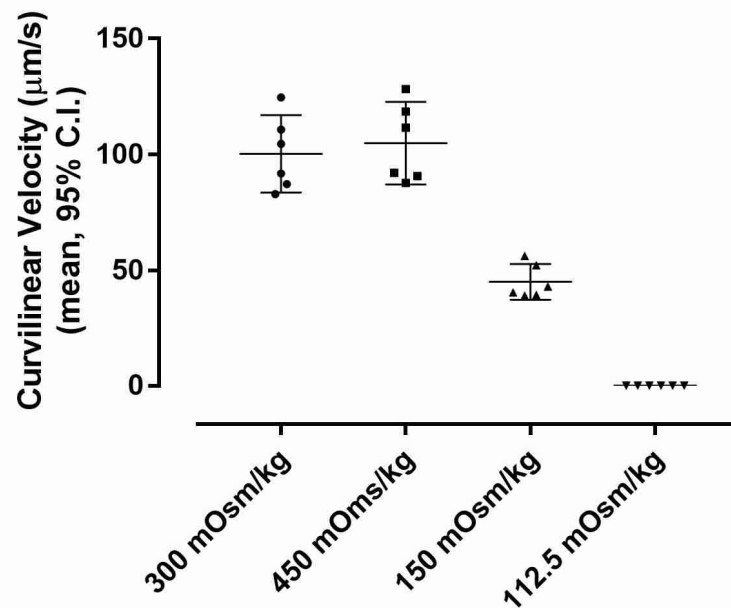


Figure 13. The curvilinear velocity (VCL) pattern of motility of sperm after exposure to varying osmolalities (300, 450, 150, and 112.5 mOsm/kg). (From Study III, Fig. 4).

Motility after hypotonic challenge following adaption to high osmolality

Spermatozoa that were adapted to an osmolality of 400 mOsm/kg and then suddenly exposed to an osmolality of 290 mOsm/kg showed a significant reduction in in curvilinear velocity and average path velocity but not in straight line velocity by CASA. The observed motility pattern of the spermatozoa under the microscope with less head displacement corresponded to the motility pattern detected by CASA. In addition, the proportion of progressively motile spermatozoa decreased after the sudden lowering in osmolality. After 60 min of incubation neither the VCL, VAP nor the progressive motility had recovered (Study III)

Hypotonic challenge and sperm tail coiling and folding

There was a general observation when assessing the spermatozoa in the microscope that the tails of the spermatozoa exposed to an hypoosmotic challenge appeared shorter. After fixation of tested aliquots inspection of individual spermatozoa revealed that the tails had undergone varying degrees of tail tip coiling or folding. The coiling was similar to, but to a lesser degree, than what occurs during the HOS test where the tails are usually completely coiled. When

osmolality was reduced from 400 to 290 mOsm/kg a median of 90% (range 75-95%) of sperm had coiled or folded tails (Fig. 8 for categories) compared to a median of 8% (range 3-15%) in the controls. In addition, for spermatozoa processed on density gradients with various osmolalities between 310 and 350 mOsm/kg (Fig. 10) and then exposed to media with an osmolality of 290 mOsm/kg the greater decrease in osmolality, the larger the proportion of spermatozoa with coiled tail tip or folded tail, in a dose-response relation (Fig. 14) (Study III).

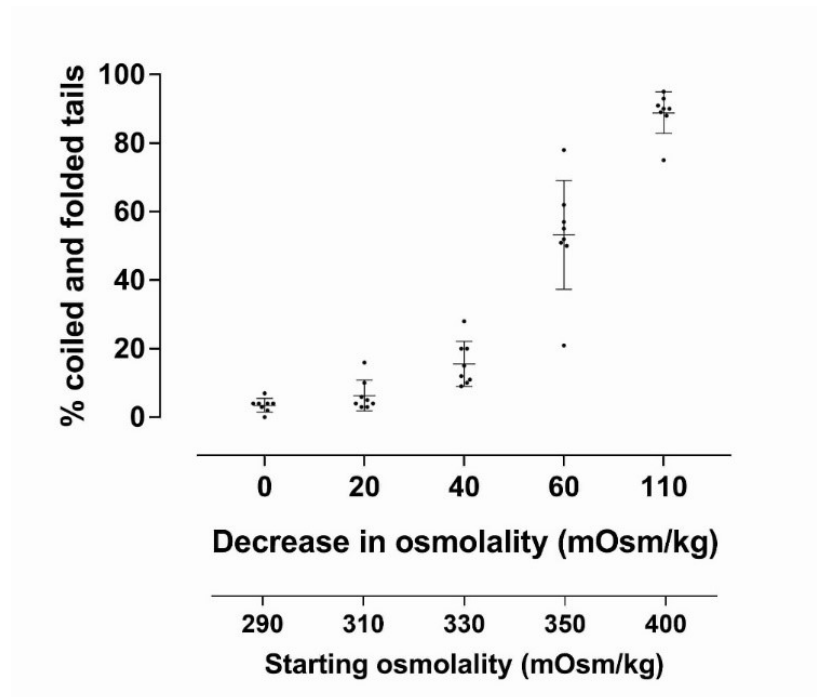


Figure 14. Graph showing the % coiled and folded sperm tails in correlation to the decrease in osmolality or hypotonic challenge they were exposed to. The added x-axis shows the initial osmolality prior to the exposure. (From Study III, Fig. 5).

4.5 LABORATORY PROCEDURES AND YIELD OF PROGRESSIVE AND DNA INTACT SPERMATOZOA BY DENSITY GRADIENT CENTRIFUGATION

Incubation at 37°C

In ejaculates stored at 37°C for 3 hours the osmolality increased from 334 mOsm/kg (range 308-380) to 366 mOsm/kg (325-422), with an average increase of 32 mOsm/kg (range 14-66). The osmolality in the density gradients was 300-310 mOsm/kg, causing an osmotic decrease (“jump”) for exposed spermatozoa. The yield of those samples after density gradient centrifugation decreased from 9.5% (2.8-17.5) to 5.3% (1.7-12.1) after extended storage at 37°C. In Fig. 15 the yield (%) is shown in relation to the decrease in osmolality, black dots at 0 hours and blank squares 3 hours later (Study IV).

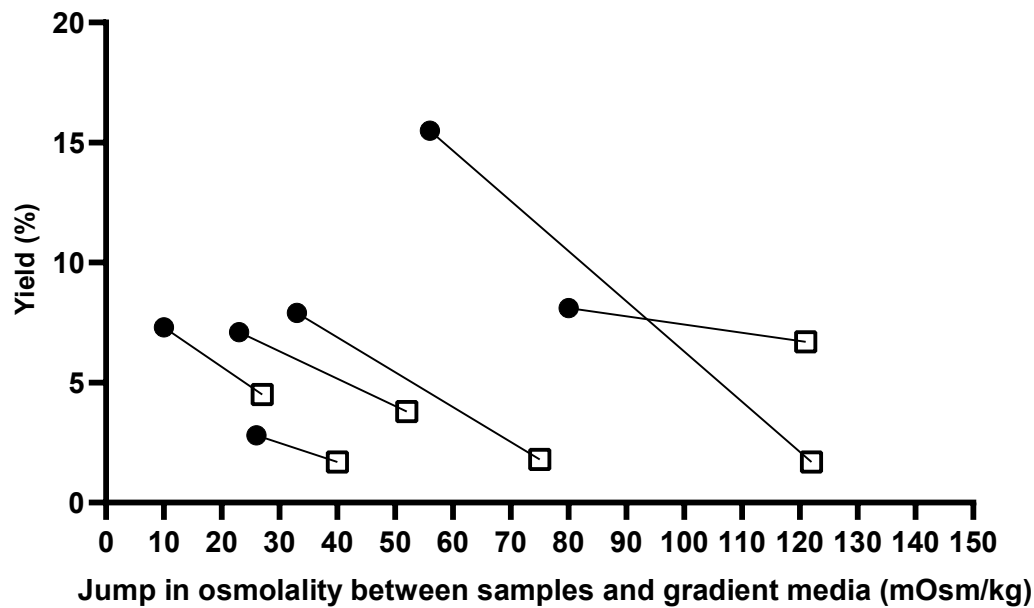


Figure 15. Yield (%) after gradient centrifugation in correlation to the decrease (“jump”) in osmolality between ejaculate and gradient at 0 hours (●) and at 3 hours (□). Ejaculates (n=7) stored at 37°C to increase osmolality. (From Study IV, Fig. 3).

Incubation at room temperature

Osmolality and yield (%) were assessed after 2 hours of storage at room temperature after liquefaction. The osmolality increased (change 37 mOsm/kg, range 12-49) and the yield decreased (change -8.2%, range: -30 to 2) (Study IV).

Incubation at room temperature after ejaculation into buffer

Ejaculates collected directly into a buffered salt solution had an osmolality of 308 mOsm/kg (range 301-315) and after two hours of incubation in vitro it was 319 mOsm/kg (314-332) (change: 11 mOsm/kg, 5-19). The yield was 23.7% (range 11.2-41.3) and after two hours of incubation 12.7% (6.2-20.0) (change -11.0, range -21 to +1) (study IV).

Defragmentation Index and High DNA stainable sperm

After 2 hours of incubation of ejaculates at room temperature, spermatozoa did not show any difference in the proportion of spermatozoa with increased DNA Fragmentation Index (DFI%) (Md 8% vs 7%, Study IV, Table II) or proportion High DNA Stainable Sperm (%HDS) (Md 11% vs. 11%, Study IV, Table II) compared to controls measured after liquefaction but before incubation (Study IV)

Ejaculates diluted with a medium with an osmolality of 300 mOsm/kg and then incubated for 2 hours at room temperature, did not show any difference in the proportion of spermatozoa with increased Defragmentation Index (DFI%) when compared to undiluted controls measured immediately after dilution (Study IV, Table 2).

Spermatozoa ejaculated directly into a buffer had lower DFI% values at 0 hours and after 2 hours of storage in vitro compared to samples diluted with buffer shortly after ejaculation, while there were not lower values for in %HDS (Study IV).

5 DISCUSSION

The method of freezing-point depression was reliable for measuring the osmolality of semen samples in the range analyzed in these experiments.

By measuring semen samples within 5 min after ejaculation one can assume that the osmolality at ejaculation is close to the same or even slightly lower. It is therefore safe to say that the osmolality of semen at ejaculation is close to isotonic to other body fluids which confirms earlier reports by (Cooper et al., 2005; Keitel & Jones, 1956; Rothschild 1960). The measured osmolality shortly after liquefaction shows a pronounced increase in osmolality and after three hours the osmolality had increased substantially which is in agreement with earlier reports (Makler et al., 1981; Polak & Daunter, 1984; Rossato et al., 2002; Velazquez et al., 1977). However, in all these earlier studies the osmolality was measured only at one time-point within 60 minutes after ejaculation, meaning that these studies only have focused on static measurements, while the present projects have focused on investigating the dynamics of osmolality in vitro by measurements of osmolality at different time-points. Experimental incubations were done to uncover intrinsic properties of the ejaculates and expose possible mechanisms for effects of changing osmolality on sperm functions and ART procedures.

In **study I**, it was hypothesized that the increase in osmolality was caused by an enzymatic degradation of macromolecules in the sample, since the osmolality was shown to be temperature dependent and that at denaturing temperature the development stopped. When in **study II** the addition of enzymatic inhibitors caused the development of osmolality to stop that evidence strongly supported the hypothesis. This theory has been presented previously (Kubicek, Lindner, & Santavy, 1959; Mann, 1964; Quinlivan, 1972).

To further elucidate if any other factors present in the ejaculate could be responsible for the increase in osmolality the investigation continued in **study II**. The sperm concentration was shown not to influence the increment in osmolality. The complete removal of sperm by centrifugation did not change the increment either. By examining samples from men that had been vasectomized and azoospermic samples it could be concluded that factors potentially leaking from sperm prior ejaculation did not influence the increment in osmolality. Factors of epididymal origin did not appear to influence the increment in osmolality since the absence of epididymal factors as in post-vasectomy samples had the same increase in osmolality as did normal samples. Since there was no relation between concentration of fructose and increase in osmolality factors of seminal vesicular origin did not appear to influence the rate of increase in osmolality. However, factors with prostatic origin did appear to have contributed to the increment in osmolality since there was a significant relation between that and concentration of zinc. The prostatic secretion is main source of enzymes that can cleave proteins and other compounds present in the seminal vesicular secretion (Kubicek et al., 1959; Lilja & Weiber, 1984; Lundquist, 1947, 1949, 1952; Mann, 1964; Mann & Lutwak-Mann, 1981; Quinlivan, 1972).

Proteolysis in semen can be counteracted by dilution of the samples (Bucht, Arver, & Sjöberg, 1986). In **study II** it was shown that diluting the samples significantly reduced the development of osmolality over time.

The sperm used for ART are most often prepared prior to fertilization by one of several methods. Two very common methods are swim-up and density gradient centrifugation. They both involve adding the sperm to a medium. These are standard cell culture media that are commercially available. The dilemma is that they often have an isotonic osmolality and sperm will potentially encounter a hypoosmotic challenge when going from an increased osmolality in the sample to an isotonic medium. How does that affect the function of the sperm?

In **study III** it was further investigated how a hypoosmotic or hyperosmotic challenge would affect the spermatozoa and their motility. It turns out that a hyperosmotic challenge for the sperm, going from 300 to 450 mOsm/kg, did not affect sperm motility. In contrast a hypoosmotic challenge for the sperm, going from 300 mOsm/kg to a lower osmolality decreased motility. Similarly, if the sperm had adapted to a higher osmolality of 400 mOsm/kg, as could happen during storage, and then were exposed to an isotonic osmolality both sperm velocity (VCL and VAP) and the proportion of progressive sperm decreased. The motility had not recovered after 1 hour. This is in agreement with an earlier observation that neither tail appearance nor motility normalized after a hyperosmotic challenge (Drevius & Eriksson, 1966). The pattern of motility changed to a less curvilinear pattern since VCL and VAP decreased whereas VSL stayed the same. In addition, the tail appearance changed in 90% of the spermatozoa that had been exposed to a hypoosmotic challenge from 400 to 290 mOsm/kg. Sperm that had adapted to an osmolality of 350, 330 or 310 mOsm/kg and then were exposed to an isotonic osmolality of 290 mOsm/kg showed a dose-response effect of increased tail tip coiling and tail folding.

These tail tip coiling and tail folding effects have been seen before, they were first mentioned in 1856 by Kölliker and then explained by Drevius in 1963. They then hypothesized that the sperm took up water and swelled up from the hypotonicity. The same effect can be seen in the HOS test (ref) where the sperm that are alive swell up and become completely round.

The osmolality of ejaculates can easily reach 330 mOsm/kg during storage in vitro for 60 min. In **Study I** 43% of ejaculates had an osmolality of at least 330 mOsm/kg, which is in concordance with earlier studies (100%: Velazquez et al 1977; 67%: Makler et al 1981). This would mean that they would swell up when added to the isotonic medium of the swim-up or density gradient. Since density gradient selects sperm based on their density the question is if the outcome of such a treatment would be affected by such an event.

In **study IV** it was investigated whether the yield, % sperm, is affected by increases in osmolality in the samples prior to selection. The results suggest that early dilution or ejaculation into a buffered solution will counteract the increase in osmolality. In addition, the yield following a density gradient centrifugation decrease with increased storage time of spermatozoa and early dilution or ejaculation into buffered solution can counteract this effect.

The results in study IV also indicated that defragmentation index (DFI%) and highly stainable DNA sperm (%HDS) did not increase after 2 h of storage after liquefaction, nor was it affected by early dilution. In contrast, ejaculation into a buffered solution seemed to decrease the DFI. However, it has been suggested that the method used in this study, flow cytometry of acridine orange stained spermatozoa, should only be used on fresh semen samples (REF jfr Barratt et al 2010??).

Motility is the main energy consuming process in human spermatozoa. Maintaining the cellular volume is also an energy consuming process that consumes ATP in spermatozoa just as in somatic cells (Cosson, 2013). Sperm exposed to increasing osmolality probably have reduced energy reserves due to the efforts to maintain the cellular volume. The energy is needed for motility since that is essential to fertilization.

6 CONCLUSION

The knowledge gained in the present projects concerns an important factor for cellular functions, osmolality, that is likely to influence the outcome of sperm selection and sperm physiological functions relevant for the result of ART.

Study I

It can be concluded that the method of freeze-point depression is a reliable and robust method of measuring osmolality in human semen samples in the range that was analyzed. Furthermore, it can be concluded that the osmolality of semen is isotonic at ejaculation and that it increases with time when stored *in vitro*. There is a large individual variability in how much osmolality changes in each sample and the change is dependent on what temperature the samples are stored at. It can be suspected that the cause of the increase is of enzymatic origin, however, this needs to be further investigated.

Study II

In this study it was concluded that the individual increase in osmolality in semen samples with time *in vitro* is not related to the concentration or mere presence of the sperm. It does however seem to be an enzymatic process that is causing the increase in osmolality and it is related to the marker for prostatic fluid, zinc. On the other hand, semen dilution reduces the increment in osmolality and could be an easy and practical way to eliminate osmotic challenges to the sperm during handling in the laboratory.

Study III

In this study it was concluded that a hypotonic challenge in osmolality will affect the motility pattern of sperm and that it causes the tails to coil and fold. The most likely pattern of events is that the spermatozoa swell due to the hypotonic challenge, and this causes the tails to coil and fold which in turn leads to a changed motility pattern. A hypertonic challenge does not result in any change of motility.

Study IV

The results in this study support the previous findings that the osmolality in semen samples increase with time of storage and that keeping the samples in 37°C make the increase more dramatic. Keeping the samples in room temperature will lower the increase in osmolality and diluting the samples soon after ejaculation will lower the increase even more. Ejaculating directly into a buffered salt solution and keeping the samples at room temperature gave the greatest effect. The results also show that defragmentation index and high stainable DNA sperm is not related to a 2 h time of storage or an increase in osmolality with the method used.

This thesis is set out to clarify that current routines for preparation of sperm prior to ART infer some challenges compared to what occurs when sperm leaves the isotonic prostatic secretion and enters the isotonic cervical mucus during intercourse. The extended durations of incubations used here to elucidate possible mechanisms were not chosen to illustrate actual

clinical procedures. However, the present findings clearly point to that all ejaculate handling *in vitro*, including liquefaction, should be as short as possible or even replaced by early ejaculate dilution or collection of ejaculates in a medium.

7 FUTURE PERSPECTIVES

The intention of this thesis is to highlight the opportunities for the improvement of methods for handling ejaculates in order to eliminate negative effects on sperm selection and overall ART outcome.

- It is important that the method for collecting ejaculates should be easy for men. An example of a more difficult ejaculate collection technique is the collection of split-ejaculates which aims to obtain a sperm-rich fraction with the potential to better pregnancy outcome. Since this method of collection minimizes the contact between the sperm and the seminal vesicular secretion it eliminates the need for liquefaction allowing almost direct selection of motile spermatozoa. However, since this method of collection is more complicated for the man, there may be a higher risk that the collection is incomplete. Early dilution or ejaculation directly into a buffered solution could constitute techniques that would be much easier for each man and still result in better control of osmolality.
- It is of great importance that the procedures for handling the ejaculate *in vitro* are not harmful to the sperm. The time between ejaculation and processing should be minimized. Therefore, early dilution of the ejaculates directly after ejaculation or the addition of a buffered salt solution to the collection container prior to collection would minimize the effect of the increasing osmolality. This would be preferable from the point of *in vitro* handling of spermatozoa for ART.
- Further studies are necessary to examine if improved osmolality handling of spermatozoa is linked to improved fertilization (number of usable blastocysts), pregnancy frequency, and healthy children born.
- With this study, the field of knowledge regarding the osmolality effects in sperm handling has been deepened. This further knowledge will allow the development of laboratory techniques which are easy to implement and practical for the laboratory personnel to perform.
- The results presented here indicate possibilities for improved laboratory osmolality handling that do not require expensive and complicated equipment. This is in concordance with international recommendations for standardized protocols that work globally.

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-Every sperm is sacred

-Monty Python's "The Meaning of life"

ANOVA Rainbow Sperm. Design: Ulrik Kvist www.zinczperm.com, Photo: Pontus Stigberg

9 REFERENCES

- Afzelius B. Electron microscopy of the sperm tail; results obtained with a new fixative. *J Biophys Biochem Cytol.* 1959;5(2):269–278. doi:10.1083/jcb.5.2.269
- Agirregoitia E1, Valdivia A, Carracedo A, Casis L, Gil J, Subiran N, Ochoa C, Irazusta J. Expression and localization of delta-, kappa-, and mu-opioid receptors in human spermatozoa and implications for sperm motility. *J Clin Endocrinol Metab.* 2006 Dec;91(12):4969-75.
- Agirregoitia E1, Subiran N, Valdivia A, Gil J, Zubero J, Irazusta J. Regulation of human sperm motility by opioid receptors. *Andrologia.* 2012 May;44 Suppl 1:578-85.
- Avidor-Reiss T, Mazur M, Fishman EL, Sindhvani P. The Role of Sperm Centrioles in Human Reproduction - The Known and the Unknown. *Front Cell Dev Biol.* 2019;7:188. Published 2019 Oct 1. doi:10.3389/fcell.2019.00188
- Barratt CL, Kay V, Oxenham SK. The human spermatozoon - a stripped down but refined machine. *J Biol.* 2009;8(7):63. doi:10.1186/jbiol1167
- Björndahl L., Kjellberg S., Kvist U. Ejaculatory sequence in men with low sperm chromatin-zinc. *Int. J Androl.* 1991, 14, pages 174–178
- Björndahl, L., Mortimer, D., Barratt, C. L. R., Castilla, J. A., Menkveld, R., Kvist, U., ... Haugen, T. B. (2010). A practical guide to basic laboratory andrology. Cambridge, UK and New York, NY: Cambridge University Press.
- Björndahl, L., & Kvist, U. (2003). Sequence of ejaculation affects the spermatozoon as a carrier and its message. *Reproductive BioMedicine Online*, 7(4), 440–448. [https://doi.org/10.1016/S1472-6483\(10\)61888-3](https://doi.org/10.1016/S1472-6483(10)61888-3)
- Bolton V. N., Warren R. E., Braude R. R. Removal of bacterial contaminants from semen for *in vitro* fertilization or artificial insemination by the use of buoyant density gradient centrifugation. *Fert. Steril.* 46:1128, 1986
- Bungum, M., Bungum, L., & Giwercman, A. (2011). Sperm chromatin structure assay (SCSA): a tool in diagnosis and treatment of infertility. *Asian J Androl*, 13(1), 69-75. <https://doi.org/10.1038/aja.2010.73>
- Bucht, E., Arver, S., & Sjöberg, H. E. (1986). Immunoextracted calcitonin from human seminal plasma constitutes different forms. *Acta Physiologica Scandinavica*, 126(2), 289–293. <https://doi.org/10.1111/j.1748-1716.1986.tb07816.x>
- Chen Q1, Duan EK. Aquaporins in sperm osmoadaptation: an emerging role for volume regulation. *Acta Pharmacol Sin.* 2011 Jun;32(6):721-4.
- Cooper TG, Yeung CH. Acquisition of volume regulatory response of sperm upon maturation in the epididymis and the role of the cytoplasmic droplet. *Microsc Res Tech.* 2003;61(1):28–38. doi:10.1002/jemt.1031
- Cooper T G, Yeung C H, Nashan D, Jockenhövel F, Nieschlag E. Improvement in the assessment of human epididymal function by the use of inhibitors in the assay of alpha-glucosidase in Seminal Plasma. *Int J Androl* 1990; 13 (4): 297-305

- Cooper, T. G., Barfield, J. P., & Yeung, C. H. (2005). Changes in osmolality during liquefaction of human semen. *International Journal of Andrology*, 28(1), 58–60. <https://doi.org/10.1111/j.1365-2605.2004.00506.x>
- Cosson, J. (2013). Chapter 1: ATP, the spermatozoa source of energy. In E. Kuester & G. Traugott 271 (Eds.), *Adenosine triphosphate : chemical properties, biosynthesis and functions in cells* (pp. 272 1-46). New York: Nova Biomedical.
- Curry MR, Millar JD, Watson PE. The presence of water channel proteins in ram and human sperm membranes. *J Reprod Fertil* 1995; 104:297–303. Mann, T. (1964). *The biochemistry of semen and of the male reproductive tract*. London, UK: Metheun & Co Ltd.
- Drevius, L. O. (1963). Spiralization in Tails of Mammalian Spermatozoa in Hypotonic Media. *Nature*, 197(4872), 1123-1124. <https://doi.org/10.1038/1971123b0> 241
- Drevius, L. O., & Eriksson, H. (1966). Osmotic swelling of mammalian spermatozoa. *Exp Cell Res*, 42(1), 136-156. [https://doi.org/10.1016/0014-4827\(66\)90327-2](https://doi.org/10.1016/0014-4827(66)90327-2)
- Drevius LO. Permeability coefficients of bull spermatozoa for water and polyhydric alcohols. *Exp Cell Res* 1971; 69: 212–6.
- Evenson D, Jost L (2001) Sperm chromatin structure assay for fertility assessment. In Robinson JP, Darzynkiewicz Z, Dobrucki J, Hyun WC, Nolan JP, Orfao A, Rabinovitch PS (eds) *Current Protocols in Cytometry*. John Wiley & Sons, Inc., Hoboken, USA.
- Faduola, P., & Kolade, C. O. (2015). Sperm chromatin structure assay results in Nigerian men with unexplained infertility. *Clin Exp Reprod Med*, 42(3), 101-105. <https://doi.org/10.5653/term.2015.42.3.101>
- Ford W.C. Glycolysis and sperm motility: Does a spoonful of sugar help the flagellum go around? *Hum. Reprod. Update*. 2006. 12, 269-274
- Ford WC, Harrison A. The role of oxidative phosphorylation in the generation of ATP in human spermatozoa. *J Reprod Fertil*. 1981 Sep;63(1):271-8.
- Fraser L R.. Fertilization promoting peptide: an important regulator of sperm functions in vivo? *Rev Reprod*. 1998 Sep;3(3):151-4.
- Guan J, Kinoshita M, Yuan L: Spatiotemporal association of DNAJB13 with the annulus during mouse sperm flagellum development. *BMC Dev Biol* 2009, 9:23.
- Haden NP1, Hickox JR, Whisnant CS, Hardy DM. Systematic characterization of sperm-specific membrane proteins in swine. *Biol Reprod*. 2000 Dec;63(6):1839-47.
- Henkel RR, Schill WB. Sperm preparation for ART. *Reprod Biol Endocrinol*. 2003;1:108. Published 2003 Nov 14. doi:10.1186/1477-7827-1-108
- Henkel R. Sperm preparation: state-of-the-art--physiological aspects and application of advanced sperm preparation methods. *Asian J Androl*. 2012;14(2):260–269. doi:10.1038/aja.2011.133

- Holstein AF1, Schulze W, Davidoff M. Understanding spermatogenesis is a prerequisite for treatment. *Reprod Biol Endocrinol*. 2003 Nov 14;1:107.
- Huggins C.B., Johnson A.A. Chemical observations on fluids of the seminal tract. *Amer. J. Physio*. 1933.
- Jeyendran R. S., Van der Ven H. H., Zaneveld L. J. D. (1992) The Hypoosmotic Swelling Test: An Update, *Archives of Andrology*, 29:2, 105-116, DOI:10.3109/01485019208987714
- Johnsen Ö, Eliasson R, Evaluation of a commercially available kit for the colorimetric determination of zinc in human seminal plasma. *Int J Androl* 1987; 10:435-440
- Jonsson M, Linse S, Frohm B, Lundwall A, Malm J: Semenogelins I and II bind zinc and regulate the activity of prostate-specific antigens. *Biochem J*. 2005. 387:447-453
- Jovine L. X-ray of fertilization. *Mol Reprod Dev*. 2011;78(1):1. doi:10.1002/mrd.21269
- Karvonen, M. J., & Malm, M. (1955). Colorimetric determination of fructose with indol. *Scandinavian Journal of Clinical and Laboratory Investigation*, 7(4), 305–307. <https://doi.org/10.3109/00365515509134669>
- Keitel, H. G., & Jones, H. S. (1956). The mineral and water composition of normal human sperm. *Journal of Laboratory and Clinical Medicine*, 47(6), 917–919.
- King LS, Kozono D, Agre P. From structure to disease: the evolving tale of aquaporin biology. *Nat Rev Mol Cell Biol* 2004; 5: 687–98.
- Kirkman-Brown JC, Barratt CL, Publicover SJ. Slow calcium oscillations in human spermatozoa. *Biochem J*. 2004;378(Pt 3):827–832. doi:10.1042/BJ20031368
- Kubicek, R., Lindner, E., & Santavy, F. (1959). Amino acids in the seminal plasma of various mammals. *Bulletin de la Societe de Chimie Biologique*, 41, 1345–1359
- Kvist U. Can disturbances in the ejaculatory sequence contribute to male infertility. *Int. J Androl*. 1991, 14, pages 389-393.
- Kölliker, A. (1856). Physiologische Studien über die Samenflüssigkeit. *Zeitschrift für Wissenschaftliche Zoologie*, VII, 201-272.
- Lilja, H., & Weiber, H. (1984). Synthetic protease inhibitors and post-ejaculatory degradation of human semen proteins. *Scandinavian Journal of Clinical and Laboratory Investigation*, 44(5), 433–438. <https://doi.org/10.3109/00365518409083834>
- Lishko PV1, Botchkina IL, Kirichok Y. Progesterone activates the principal Ca²⁺ channel of human sperm. *Nature*. 2011 Mar 17;471(7338):387-91.
- Litvin TN1, Kamenetsky M, Zarifyan A, Buck J, Levin LR. Kinetic properties of "soluble" adenylyl cyclase. Synergism between calcium and bicarbonate. *J Biol Chem*. 2003 May 2;278(18):15922-6.

- Lundquist, F. (1949). Aspects of the biochemistry of human semen. *Acta Physiologica Scandinavica*, 19(Suppl 66), 1–108. <https://doi.org/10.1111/j.1748-1716.1949.tb00552.x>
- Mahadevan M, Baker G: Assessment and preparation of semen for in vitro fertilization. In: *Clinical In Vitro Fertilization* Edited by: Wood C, Trounson A. Springer-Verlag, Berlin; 1984:83-97.
- MacLeod, J., & Hotchkiss, R. S. (1942). The distribution of spermatozoa and of certain chemical constituents in the human ejaculate. *Journal of Urology*, 48(2), 225–229.
- Magli MC1, Van den Abbeel E, Lundin K, Royere D, Van der Elst J, Gianaroli L; Committee of the Special Interest Group on Embryology. Revised guidelines for good practice in IVF laboratories. *Hum Reprod*. 2008 Jun;23(6):1253-62.
- Makler, A., David, R., Blumenfeld, Z., & Better, O. S. (1981). Factors affecting sperm motility. VII. Sperm viability as affected by change of pH and osmolality of semen and urine specimens. *Fertility and Sterility*, 36(4), 507–511. [https://doi.org/10.1016/S0015-0282\(16\)45802-4](https://doi.org/10.1016/S0015-0282(16)45802-4)
- Mann, T. (1964). *The biochemistry of semen and of the male reproductive tract*. London, UK: Methuen & Co Ltd.
- Mann, T., & Lutwak-Mann, C. (1981). *Male reproductive function and semen*. Berlin and Heidelberg, Germany: Springer-Verlag GmbH & Co.
- Marmar JL, Katz S, Praiss DE, DeBenedictis TJ. Values for zinc in whole semen, fractions of split ejaculate, and expressed prostatic fluid. *Urology*. 1980 Nov;16(5):478-80.
- Monné M1, Jovine L. A structural view of egg coat architecture and function in fertilization. *Biol Reprod*. 2011 Oct;85(4):661-9. doi: 10.1095/biolreprod.111.092098. Epub 2011 Jun 29.
- Mortimer D: Sperm Transport in the Human Female Reproductive Tract. In: *Oxford Reviews of Reproductive Biology* Volume 5. Edited by: Finn CA. Oxford University Press, Oxford; 1989:30.
- Mortimer, S. T., van der Horst, G., & Mortimer, D. (2015). The future of computer-aided sperm analysis. *Asian J Androl*, 17(4), 545-553. <https://doi.org/10.4103/1008-682X.154312>
- Noiles EE, Mazur P, Watson PF, Kleinhans FW, Critser JK. Determination of water permeability coefficient for human spermatozoa and its activation energy. *Biol Reprod* 1993; 48:99–109.13.
- Noiles EE, Thompson KA, Storey BT. Water permeability, L-p, of the mouse sperm plasma membrane and its activation energy are strongly dependent on interaction of the plasma membrane with the sperm cytoskeleton. *Cryobiology* 1997; 35:79–92
- O'Donnell L., Nicholls P.K., O'Bryan M., Spermiation. *Spermatogenesis* 2011 jan-mars;1(1):14-35.
- O'Donnell L, Stanton P, de Kretser DM. Endocrinology of the Male Reproductive System and Spermatogenesis. [Updated 2017 Jan 11]. In: Feingold KR, Anawalt B, Boyce

- A, et al., editors. Endotext [Internet]. South Dartmouth (MA): MDText.com, Inc.; 2000-. Available from: <https://www.ncbi.nlm.nih.gov/books/NBK279031/>
- Petrunkina AM, Jebe E, Töpfer-Petersen E. Regulatory and necrotic volume increase in boar spermatozoa. *J Cell Physiol.* 2005;204(2):508–521. doi:10.1002/jcp.20317
- Polak, B., & Daunter, B. (1984). Osmolarity of human seminal plasma. *Andrologia*, 16(3), 224–227. <https://doi.org/10.1111/j.1439-0272.1984.tb00269.x>
- Pousette A, Akerlöf E, Rosenborg L, Fredricsson B: Increase in progressive motility and improved morphology of human spermatozoa following their migration through Percoll gradients. *Int J Androl* 1986, 9:1-13
- Quinlivan, W. L. (1972). The effect of temperature on the proteins of human seminal plasma during storage. *Fertility and Sterility*, 23(3), 163–167. [https://doi.org/10.1016/S0015-0282\(16\)38820-3](https://doi.org/10.1016/S0015-0282(16)38820-3)
- Rodríguez-Martínez H1, Iborra A, Martínez P, Calvete JJ. Immunoelectronmicroscopic imaging of spermadhesin AWN epitopes on boar spermatozoa bound in vivo to the zona pellucida. *Reprod Fertil Dev.* 1998;10(6):491-7.
- Rodríguez-Martínez H1, Kvist U, Ernerudh J, Sanz L, Calvete JJ. Seminal plasma proteins: what role do they play? *Am J Reprod Immunol.* 2011 Jul;66 Suppl 1:11-22.
- Rothschild, V. (1960). Freezing point depression of human seminal plasma. *Journal of the National Medical Association*, 52, 6–7.
- Rossato, M., Di Virgilio, F., & Foresta, C. (1996). Involvement of osmosensitive calcium influx in human sperm activation. *Molecular Human Reproduction*, 2(12), 903–909. <https://doi.org/10.1093/molehr/2.12.903>
- Ruiz-Pesini E., Díez-Sánchez C., López-Pérez M., Enríquez J.A. The role of the mitochondrion in sperm function: Is there a place for oxidative phosphorylation or is this a purely glycolytic process? *Curr. Top. Dev. Biol.* 2007;77:3-19.
- Singer S. J., Nicholson G. L., The fluid mosaic model of the structure of cell membranes. *Science* 1972;175 (4023), 720-731. DOI: 10.1126/science.175.4023.720
- Talbot P, Chacon RS. Ultrastructural observations on binding and membrane fusion between human sperm and zona pellucida-free hamster oocytes. *Fertil Steril.* 1982;37(2):240–248. doi:10.1016/s0015-0282(16)46047-4
- Tamburrino L1, Marchiani S, Minetti F, Forti G, Muratori M, Baldi E. The CatSper calcium channel in human sperm: relation with motility and involvement in progesterone-induced acrosome reaction. *Hum Reprod.* 2014 Mar;29(3):418-28.
- Velazquez, A., Pedron, N., Delgado, N. M., & Rosado, A. (1977). Osmolality and conductance of normal and abnormal human seminal plasma. *International Journal of Fertility*, 22(2), 92–97.
- World Health Organization (2010). WHO laboratory manual for the examination and processing of human semen (5th ed.). Geneva, Switzerland: World Health Organization.

- Yanagimachi R: Mammalian fertilization. In: The Physiology of Reproduction Volume 1. Edited by: Knobil E, Neill JD, Ewing LL, Markert CL, Greenwald GS, Pfaff DW. Raven Press, New York; 1988:135-185.
- Yeung CH, Barfield JP, Cooper TG. Physiological volume regulation by spermatozoa. *Mol Cell Endocrinol.* 2006;250(1-2):98–105. doi:10.1016/j.mce.2005.12.030
- Yeung CH, Callies C, Tüttelmann F, Kliesch S, Cooper TG. Aquaporins in the human testis and spermatozoa - identification, involvement in sperm volume regulation and clinical relevance. *Int J Androl.* 2010 Aug 1;33(4):629-41
- Yudin AI, Generao SE, Tollner TL, Treece CA, Overstreet JW, Cherr GN. Beta-defensin 126 on the cell surface protects sperm from immunorecognition and binding of anti-sperm antibodies. *Biol Reprod.* 2005;73(6):1243–1252. doi:10.1095/biolreprod.105.042432